# American Journal of
# Veterinary Research


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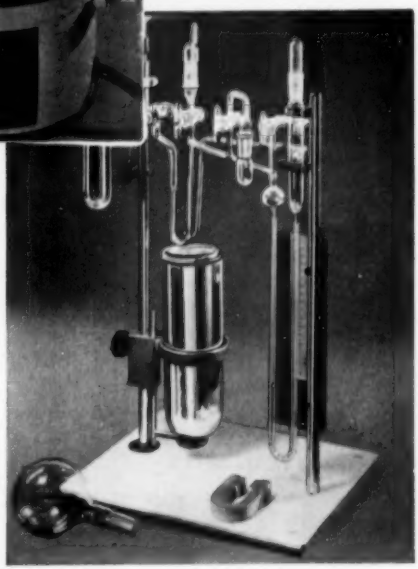

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VOL. XVIII OCTOBER, 1957 No. 69


## Studies on Avian Encephalomyelitis. I. Egg Adaptation of the Virus


F. W. SUMNER, B.S.; E. L. JUNGHERR, Vet. Dipl., D.M.V.; R. E. LUGINBUHL, M.S.

*Storrs, Connecticut*


VARIOUS ATTEMPTS have been made to adapt the virus of avian encephalomyelitis (AE) to the embryonating chicken egg. Van Roekel *et al.*[12,13] showed that chicks hatched from eggs inoculated at the beginning of the incubation period, or chicken embryos inoculated at 10 to 12 days of age, developed the disease, and also showed that hatchability of the inoculated eggs was lowered.

Kligler and Olitsky [7] and Jungherr and Minard [4] found that the chorioallantois was not an effective route for transmitting the disease to embryos. The latter authors were successful in hatching chicks showing signs of AE from chicken embryos inoculated by various routes, namely, the yolk sac, intravenously, and intracerebrally. Feibel *et al.*[3] were unsuccessful in producing lesions on the chorioallantoic membrane with $10^{4.4}$ minimum chick infective doses of virus. The same quantity of virus failed to infect after intra-amniotic injection. Repeated zig-zag, chicken embryo passages did not alter the virus sufficiently to permit adaptation by the amnioallantoic route. Nine- or 10-day-old embryos, inoculated by the intracerebral route by Hammar,[6] had a lower rate of hatchability than embryos inoculated with normal tissue suspensions. This route of inoculation produced a high percentage of incidental deaths.

Since Feibel *et al.*[3] found that the intraocular (intrabulbar) route was effective in transmitting the disease to susceptible adult chickens, and in view of the delicacy of the intracerebral route for chicken embryos, an attempt was made to adapt the virus to the chicken embryo using the former route. This work is described here.

### MATERIALS AND METHODS

*Equipment.*—A motor with a drill attachment and a stone abrasive disc about ½ inch in diameter was used to cut the egg shells for inoculation. Sterile mineral oil was used to clarify the shell membrane in order to see the embryo. A 0.25-ml. tuberculin syringe with a 1.5-inch, 26-gauge needle was used for inoculation. The eggs were resealed with Scotch cellophane tape.

*Diluent.*—Sodium phosphate buffered, physiological saline solution, at a pH of 7.2, served as the diluent.

*Embryos.*—Nine- to 11-day-old embryos were used. Our regular source of eggs was the poultry department of the University of Connecticut. The breed was Columbian. Later this flock was replaced by a four-way cross breed.

*Inoculum.*—The virus (our strain A37020) used for this work was received as the 135th chick passage from Dr. Henry Van Roekel of the University of Massachusetts and had undergone 15 chick passages in this laboratory. It was prepared by adding 9 ml. of diluent to each gram of infected brain. The mixture was put in a glass jar partially filled with glass beads and shaken in a Kahn shaker at 4 C. for two hours (Feibel *et al.*[3]). It was then dispensed in tubes and frozen at −20 C. When needed, the material was thawed and centrifuged at 2,000 r.p.m. for ten minutes. The supernatant fluid was used as the source of virus. The same procedure was employed for preparing embryonic tissues for inoculation, but the virus suspension consisted of a mixture of embryonic brain and eye.

*Inoculation Procedure.*—The eggs were candled and an area about 1 cm. in diameter was marked off over the embryo above the air cell. This area of the shell was cut through with the abrasive disc without injury to the shell membrane. The inocu-


From the Department of Animal Diseases, Storrs Agricultural Experiment Station, University of Connecticut, Storrs.


References to all literature cited will be found at the end of the second paper, pp. 720-723.


This investigation was supported in part by grant B-171 (C4) from the National Institutes of Health, U.S.P.H.S., and a grant from the American Poultry and Hatchery Federation.


As this manuscript was being prepared for publication, an article by Wills and Moulthrop [14] appeared, describing chicken embryo propagation of avian encephalomyelitis virus by the yolk sac route.

lation was done in a partially darkened room. Just prior to inoculation, the cut area was swabbed with 70 per cent ethyl alcohol and that portion of the shell and its membrane were removed. A microscope substage lamp * was set up so that the egg to be inoculated could be set on the lens and the operator could look into it through the opening. A drop of sterile mineral oil was then placed on the inner shell membrane. The candling lamp was turned on and inoculation was accomplished with a sharp thrust of the needle into the eye of the embryo. The amount of the inoculum was 0.05 ml. Finally, the egg was resealed with Scotch tape and reincubated at 37 C. The eggs were candled twice daily, once in the morning and again late in the afternoon.

*Controls.*—Both inoculated and noninoculated controls were used. The inoculated controls received either buffered physiological saline solution or a $10^{-1}$ dilution of normal embryonic brain and eye material.

*Virus-Neutralization Test.*—The virus-dilution method was employed. The virus was diluted in tenfold serial dilutions from 1:5 to 1:500,000 so that, when an equal amount of undiluted serum was added, final virus dilutions of $10^{-1}$ through $10^{-6}$ were obtained. No preliminary incubation of this mixture was practiced. Titration end points were calculated according to the method of Reed and Muench.[8]

*Chorioallantoic Chamber* (CAC) *Route.*—The egg was candled and a small hole drilled about 5 mm. above the base of the air cell. The hole was swabbed with tincture of iodine and inoculation accomplished by piercing the chorioallantoic membrane with a ½-inch, 26-gauge needle to a depth of about ¼ inch. Previous experience had indicated that the amniotic sac was not reached by this method. After injection of 0.2 cc. of inoculum, the hole was swabbed with 70 per cent ethyl alcohol and resealed with Duco aeroplane cement.

## RESULTS

From the first to the sixth day after inoculation, the embryos appeared essentially normal on candling. From the seventh to the tenth day, an increasing sluggishness was observed until there was no movement at all and the embryos finally died. On opening, the embryos appeared stiff and emaciated. The eyes appeared normal.

In 15 subsequent embryo passages, the same pattern of embryo changes was seen. Not all embryos died prior to the termination of the incubation period. Because of this, the test was allowed to continue until death or for nine days postinoculation. There were varying intensities of the above described signs, especially when titrations were done. To standardize the reading of the test, three degrees of embryonic involvement were recognized, namely: *(a)* sluggishness, if the embryo could not move its legs but could still swallow; *(b)* inactivity, if it could not move at all but was still alive as determined by persistent heartbeat; and *(c)* death, if no chorioallantoic veins were visible on candling. None of the control embryos showed such signs. The corresponding histopathological changes in the embryo have been described by Jungherr *et al.*[5]

Virus suspensions of brain and eye contained from $10^{5.9}$ to $10^{5.8}$ embryo infective doses (e.i.d.$_{50}$) in 0.05 ml. of inoculum. In separate titrations made on brain, eye, and amnioallantoic fluid (AAF) from infected embryos, $10^{5.7}$, $10^{5.2}$, and $10^{3.9}$ e.i.d.$_{50}$ were obtained, respectively. The virus was also present in the embryonic viscera.

Attempts were made at cultivating the virus in the CAC. Undiluted, infected AAF from intraocularly inoculated embryos served as the inoculum. The pattern of changes was the same as was seen when using the intraocular route. Fifteen such passages were made. Infected AAF was titered intraocularly to test for the presence of the virus at two, four, six, and eight days after inoculation. Virus was present on each day on which tests were made (table 1), the maximum amount ($10^{3.9}$ e.i.d.$_{50}$) being found on the eighth day, which fact indicated lower virus potency than was the case for intraocular passage.

After the egg source was changed from the original poultry department flock to the four-way cross flock the virulence of the virus appeared to decrease and infection by the CAC no longer occurred. The decrease in pathogenicity of the virus for embryos was also observed on intraocular titration of infected brain and eye suspensions although complete loss of potency did not ensue.

Titration of the virus in embryos from

**TABLE 1—Intraocular Titration of Amnioallantoic Fluid-Propagated AE Virus, Harvested at Different Days Postinoculation**

| Day harvested | Dilutions of AAF | | | | | | |
|---|---|---|---|---|---|---|---|
| | $10^{0}$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | e.i.d.$_{50}$ |
| 2 | 3/4* | 3/3 | 1/2 | 1/4 | 0/4 | ..... | $10^{2.9}$ |
| 4 | 2/4 | 1/2 | 3/4 | 1/4 | 0/3 | ..... | $10^{2.9}$ |
| 6 | 3/4 | 4/4 | 0/3 | 0/4 | 0/4 | 0/4 | $10^{1.4}$ |
| 8 | 3/3 | 3/3 | 3/4 | 2/4 | 1/4 | 0/4 | $10^{2.9}$ |

* Number of embryos showing signs total inoculated.

* Manufactured by the American Optical Co., Buffalo, N. Y.

**TABLE 2—Comparison of the AE Virus-Neutralization Test in Chicks and in Chicken Embryos**

| Dilution | Chicks | | Embryos | |
|---|---|---|---|---|
| | Virus | Virus and serum | Virus | Virus and serum |
| $10^{-1}$ | 10/10 | 9/9 | 4/4 | 1/4 |
| $10^{-2}$ | 10/10 | 8/9 | 4/4 | 2/4 |
| $10^{-3}$ | 8/10 | 6/10 | 4/4 | 1/4 |
| $10^{-4}$ | 10/10 | 2/10 | 4/4 | 1/3 |
| $10^{-5}$ | 2/10 | 0/10 | 3/4 | 0/4 |
| $10^{-6}$ | ..... | ..... | 2/4 | 1/4 |
| $10^{-7}$ | ..... | ..... | 0/4 | 0/3 |
| Titer | $10^{-4.5}$ | $10^{-2.1}$ | $10^{-5.8}$ | $10^{-2.6}$ |
| ND | ..... | 27 | ..... | 5,700 |

ND = Neutralizing doses.

six other sources showed the same effect. Results of titrations appeared erratic, no gradient of embryonic infection with graduated doses of virus being evident in embryos from these flocks, as distinct from that seen in embryos from the original flock.

To show that AE virus was present after embryonic passage, pathogenicity tests were run in chicks using infected embryo tissues. Birds inoculated intracerebrally, as well as intramuscularly and by the wing-web method, became ataxic seven days post-inoculation. Brains of affected birds showed lesions of AE on microscopic examination.

A further control consisted of making serological tests. Serums from birds which had recovered from experimentally induced AE inhibited infection in embryos and contained from 500 to 5,700 neutralizing doses. The virus-neutralization (VN) test was more sensitive in embryos than it was in chicks; one serum contained 5,700 protective doses for embryos and only 27 for chicks (table 2), another contained 4,500 protective doses for embryos and 13 for chicks.

## Discussion

The seemingly complicated technique used for cultivating the virus of AE by the intraocular injection of the chicken embryo became quite simple and rapid with practice. For example, 4 or 5 embryos could be prepared simultaneously and inoculated consecutively without interruption. Cultivation by the CAC route was also possible but titers were not so high as for intraocular inoculation. Fifteen CAC passages have been carried out so far.

Variations in titers of a standard AE virus in embryos from different sources have been observed. It is known that in certain diseases of fowl, immunity is passed from the hen to the embryo. In view of this, it would not be difficult to understand why the titer of AE virus should vary among embryos from various flocks of chickens. The immunization results reported by Schaaf and Lamoreux[9] would likewise support a concept of passive immunity in infected flocks. The differences in resistance of chicken embryos from various sources to AE virus propagation will be the subject of another report.

In view of the variations in titers observed, it can be seen that a prerequisite for immunological studies is a supply flock, the embryos of which show a high virus titer and thereby indicate susceptibility of the flock.

For research work with AE virus, embryos offer certain advantages over chicks. Eggs are more easily cared for and less expensive. The VN test seems to be more sensitive in embryos than in chicks. Time is saved, nine days being the length of time needed for a test in eggs while 14 to 28 days is required for a similar test in chicks. The large amount of virus of known potency provided by embryos may be useful for vaccine studies.

## Summary

Egg adaptation of the virus of avian encephalomyelitis has been achieved by intraocular inoculation of chicken embryos. Convalescent serum from artificially infected birds protected embryos against infection to a greater extent than it did chicks. Nine days were needed for a test in embryos as compared with 14 to 28 days in chicks. Most of the flocks tested produced partially resistant embryos, indicating that, for successful laboratory work, the embryo supply flock must be chosen with care. It was possible to infect the embryo via the chorioallantoic chamber with infected brain and eye suspensions and infected amnioallantoic fluid from other embryos.

# Studies on Avian Encephalomyelitis. II. Flock Survey for Embryo Susceptibility to the Virus

F. W. SUMNER, B.S.; R. E. LUGINBUHL, M.S.; E. L. JUNGHERR, Vet. Dipl., D.M.V.

*Storrs, Connecticut*

IN STUDIES with the egg-adapted strain of avian encephalomyelitis (AE), it was found that the end points of virus titrations in embryos differed markedly with eggs from different sources.[10] This suggested a change in the pathogenicity of the virus since high titers had been obtained consistently during 13 passages, over a period of eight months, in embryos from the original source. Errors in technique and storage were believed to be of minor importance in explaining this phenomenon. The lowered pathogenicity was tentatively ascribed to differences in susceptibility of the embryos. To test this hypothesis, 36 eggs were collected from each of more than 100 flocks of chickens. These eggs were incubated for 11 days at 37 C., and the embryos challenged with graduated doses of the virus.

## MATERIALS AND METHODS

*Virus.*—The strain of AE virus used was that adapted to the chicken embryo by intraocular injection and stored as described.[10]

*Virus Titration.*—Tenfold serial dilutions of the virus from $10^{-1}$ through $10^{-6}$ were prepared in phosphate buffered saline solution (PBS). Six, 11-day-old chicken embryos were used per dilution. The amount of the inoculum was 0.05 ml. per intraocular injection.[10] Deaths occurring earlier than six days postinoculation were classified as incidental. Reading of the test was based on degrees of embryonic inactivity,[10] and end points were calculated according to the method of Reed and Muench.[8]

*Embryos.*—Hatching eggs were collected from Connecticut and Illinois. In Connecticut, two hatcheries and nine miscellaneous flocks supplied the eggs. One hatchery received eggs from 18 supply flocks and reported no difficulties with AE. The other hatchery also had 18 contributing flocks and reported that AE-affected chicks had been produced for two months prior to testing. It was not possible to determine exactly which flocks supplied the hatchery responsible for producing the affected chicks. For the nine miscellaneous flocks, there was no definite history of AE.

In Illinois, one hatchery supplied eggs which had been produced by 72 flocks. At the time of the tests (Sept., 1956), 14 of these flocks were being used for hatchery purposes, ten of which had produced AE-affected chicks. Forty-seven of the flocks were composed of pullets. Since chicks had not been hatched from these flocks, no AE history could be obtained. The remaining 11 flocks, which represented the hatchery's pedigreed stock, likewise had no known AE history.

*Virus-Neutralization Test.*—Virus dilutions were prepared as stated.[10] Of the materials tested for antibodies, serum was used undiluted, and yolk as a 1:1 dilution in PBS. Adult serum was pooled from birds of the flock being tested, and serum from 2-day-old chicks was pooled from 20 birds. Equal amounts of yolk from 20 eggs formed the yolk pools.

## RESULTS

Titration end points of the virus of AE in embryos from 119 flocks of chickens ranged from $10^{-0.9}$ to $10^{-5.8}$, examples of which are shown (table 1). The outstanding feature of these titrations was the excessive number of what Reed and Muench [8] termed "'accidental' survivals and deaths," when titers were low. In sample titration 4 (table 1), 1 of 3 of the embryos inoculated showed signs of AE, such as sluggishness (defined in the preceding paper), at a virus dilution of $10^{-2}$; none of 4 did so at a dilution of $10^{-3}$; 1 of 4 at a dilution of $10^{-4}$; and 2 of 3 at a dilution of $10^{-5}$. In the few cases where uniform virus growth occurred, titers of $10^{-5}$ or higher were obtained (sample titration 1, table 1).

**TABLE 1—Sample Titrations of AE Virus in Embryos of Different Degrees of Susceptibility**

| Sample | Virus dilution | | | | | | Titer |
|---|---|---|---|---|---|---|---|
| | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | |
| 1 | 4/4 | 4/4 | 4/4 | 4/4 | 3/4 | 3/4 | 5.8 |
| 2 | 4/4 | 4/4 | 4/4 | 1/3 | 1/2 | .... | 4.0 |
| 3 | .... | 1/2 | 1/4 | 0/4 | 3/4 | .... | 3.0 |
| 4 | .... | 1/3 | 0/4 | 1/4 | 2/3 | .... | 2.5 |
| 5 | 1/3 | 0/2 | 1/4 | 0/4 | 1/3 | .... | 1.4 |
| 6 | 1/6 | 2/5 | 0/5 | 1/5 | 0/5 | .... | 0.9 |

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The authors are indebted to Helene P. Matterson for drawing the graphs.

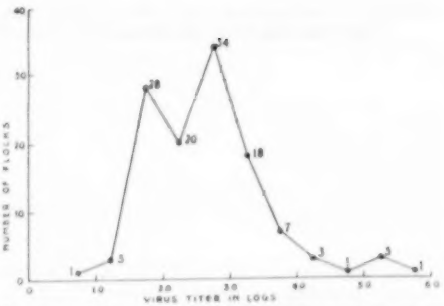
To avoid a complicated table, the results of titrations of the virus in embryos from 119 flocks are summarized (graph 1). Of these titrations, 54 had end points between $10^{-2}$ and $10^{-3}$, four had end points as high as $10^{-5}$, and only one had an end point below $10^{-1}$.

Fourteen of the flocks supplying embryos (all from Illinois) had histories as to their AE status. Ten flocks had produced AE-affected chicks. Four had not, but two of them supplied embryos in which virus titers of $10^{-4.5}$ or above were obtained. None of the ten flocks which had produced AE-affected chicks supplied embryos in which the virus titer was higher than $10^{-3.5}$. At the time eggs were collected for the tests, most of the ten flocks were still producing AE-affected chicks; those that were not had only recently stopped.

Since the above described results indicated that the decrease in virus pathogenicity first observed was due to variations in embryo susceptibility, immunological studies on this point seemed advisable. These studies, concerning transference of immune bodies from hens to their progeny by way of yolk, were based in part on the work of Brandly *et al.*[1] on Newcastle disease, and Jungherr and Terrell[6] on infectious bronchitis. Virus-neutralization tests on serum from two flocks which produced embryos of widely separated degrees of susceptibility to the virus, and on yolk and serum from their progeny, were conducted, the results of which are shown (table 2).

In embryos from one of the flocks (A), virus titers of $10^{-4}$ or higher were obtained, while in embryos from the other (B), titers were no higher than $10^{-1.8}$. Yolk from eggs produced by flock A, and adult serum from this flock, did not inhibit virus growth. Conversely, yolk from eggs produced by flock B, serum from 2-day-old flock B progeny, and flock B adult serum inhibited virus growth.

Immunological studies were not yet completed when it was observed that flock A had started producing embryos of increasing resistance to infection with AE virus (graph 2). From the history of this flock, it was found that, just prior to the time the eggs which first gave the lowered virus titer were produced, a drop in egg production had occurred (graph 2). Virus titers in embryos from this flock continued on a downward trend.



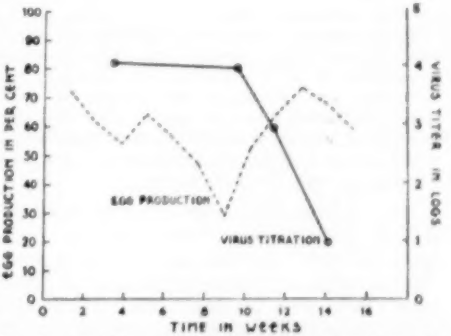

**Graph 1—Titers of avian encephalomyelitis virus in embryos from 119 flocks. Figures on curve indicate number of flocks representing approximate titer range.**

Chicks from flock A, which were to be used for pathogenicity tests, were hatched from eggs produced during the lowest level of egg production. They were all inoculated intracerebrally with AE virus. At four days postinoculation, 1 chick showed signs of AE. Since this premature development of signs was unusual, the chick was killed and a brain section was made which showed histopathological changes characteristic of AE.

## Discussion

Evidence gathered from 119 virus titrations in embryos, from that number of flocks, showed that most flocks produced embryos somewhat resistant to infection with AE virus. This may account for the lack of success reported by other workers[2-4, 7, 12, 13] in attempts to cultivate the virus in the embryonating chicken egg.

Preliminary immunological studies indi-




**Graph 2—Avian encephalomyelitis virus titers in embryos from a flock which experienced a dip in egg production.**

**TABLE 2—Comparative AE Virus Titrations with Materials from Flocks A and B**

| | Virus titration | |
|---|---|---|
| | Flock A | Flock B |
| Embryos | $10^{-6.2}$ | $10^{-1.5}$* |
| Yolk, unincubated eggs | † | $10^{-1.5}$ |
| Yolk, 9-day-old embryos | $10^{-6.0}$ | $10^{-1.7}$ |
| Serum, 2-day-old chicks | † | $10^{-1.0}$ |
| Serum, adult | $10^{-6.5}$ | $10^{-2.8}$ |

* All titrations except this one were done in embryos from flock A. † Not done.

cated that variability of AE-virus titrations in embryos from different sources might be due to parental immunity.

Evidence supporting this hypothesis follows:

*a*) Virus titers in embryos from flock A regularly reached $10^{-6}$ and higher. No neutralization of the virus by yolk from eggs produced by this flock, or by adult serum, took place.

*b*) Virus titers in embryos from flock B were regularly low ($10^{-1.5}$ or lower). Neutralization of the virus occurred with yolk from eggs produced by this flock, serum from 2-day-old progeny, and serum from adults.

*c*) Flock A experienced a drop in egg production, after which continually decreasing virus titers were obtained in its embryos (graph 2). Since Taylor *et al.*[11] reported that infection of laying flocks with AE may be accompanied by a drop in egg production, it is postulated that the increased resistance of flock A embryos was due to immunity of the parent stock developed in response to a natural occurrence of this disease. That the egg production drop was caused by an AE infection is further supported by the fact that a chick, hatched from an egg produced during the lowest level of egg production, which was being used in an AE pathogenicity test, came down with signs of AE at four days' postinoculation. After intracerebral inoculation, it has usually taken at least seven days for signs of AE to develop and the infection was believed to have been caused by passage of the virus through the egg.

*d*) In embryos from flocks of chickens which were known to have produced AE-affected chicks, virus titers of $10^{-5.5}$ or lower were obtained. No AE history was evident in flocks which produced embryos allowing titers as high as $10^{-6}$. The line of division between resistant and susceptible flocks is not known, and the actual incidence in the field may depend on risk of exposure.

The passive immunity to AE apparently differs from passive immunity to Newcastle disease. In the latter instance, immunity does not seem to protect embryos until they are 15 days old.[1] Similarly, eggs containing passive antibodies for infectious bronchitis are not appreciably more resistant to artificial infection than eggs lacking these antibodies.[6] It is possible that passive immunity has more time to exert an effect in AE, since the incubation period in eggs is at least six days, as compared with three days for the virus of Newcastle disease and that of infectious bronchitis. More work is needed on this subject.

It is not known what embryonic virus titer indicates protection against AE. According to some poultrymen, a flock of chickens which has produced AE-infected chicks once will not do so again. Titration end point alone is not sufficient to demonstrate protection. As shown (table 1), there was an overbalance of "'accidental' survivals and deaths"[8] when titers were low. The significance of this overbalance is under investigation. These findings seem to indicate that, in an immune flock, not all hens are immune to a degree measurable by this test.

The relatively low titers obtained in embryos, from a majority of flocks, seem to indicate that infection with AE virus is widespread in the field. The fact that most flocks tested (69%) had no history of AE, but produced embryos resistant to the virus, supported the findings of Taylor *et al.*[11] who pointed out that flock infection in adult birds is mild.

## SUMMARY

The virus of avian encephalomyelitis (AE) was titrated in embryos from 119 flocks. Only four flocks produced embryos which permitted uniform virus growth to titers of $10^{-5}$. Virus-neutralizing substances were demonstrated in adult serum, yolk, and serum of progeny from a flock whose embryos gave virus titers of $10^{-1.5}$ or lower.

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# Pathological Changes of an *Escherichia Coli* Infection in Chickens and Turkeys

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CHRONIC RESPIRATORY DISEASE (CRD) is a relatively common respiratory infection of chickens and turkeys caused by a pleuropneumonia-like organism (PPLO).[12] The uncomplicated PPLO infection is characterized by high morbidity, low mortality, and a chronic course. In combination with other organisms, such as *Escherichia coli*, mortality may be greatly increased. Field infections of *E. coli* complicating CRD are usually characterized by short or medium duration and 0.5 to 10.0 per cent mortality. Losses usually occur among birds of 4 to 12 weeks of age but may occur in birds of any age. Anorexia, ruffling of the feathers, and a temperature of 108.0 to 110.4 F. occur and, occasionally, there is unilateral blindness associated with hypopyon; in severe, acute infection, there is often a yellowish diarrhea. The duration of infection in individual birds is short and usually only a few birds are sick at one time. Many infected birds do not die, but are injured to the extent that they become unprofitable to the grower. The gross lesions are pericarditis, perihepatitis, aerosacculitis, and salpingitis.

Wasserman [5] and Fahey [16] have reported the isolation of *E. coli*, together with other bacteria, from field cases of pericarditis associated with CRD. The experimental production of pericarditis in birds, by coliform bacteria, has been reported by David,[4] Twisselman,[15] Bornstein,[1] and Gross.[6]

## MATERIALS AND METHODS

*Strains of Escherichia Coli.*—Five serological types of *E. coli* have been isolated from the pericardial sacs of chickens or turkeys from 30 farms. Only three of these types were taken from more than two sources. They have been typed by the Communicable Disease Center, U. S. Public Health Service, and belong to "O" groups 1, 2, and 78. These strains, all of which were used in these studies, were of approximately the same pathogenicity. Strains were maintained as nutrient agar stab cultures and were inoculated into birds as 18- to 24-hour tryptose broth cultures.

*Strains of CRD Agent.*—A strain of PPLO was isolated from a Virginia chicken and had been passed for several years in 7-day-old embryonating eggs by the yolk sac method. This strain was free from bacteria and no evidence of viral agents could be demonstrated.

Chickens between 3 and 8 weeks of age were inoculated in the lesser abdominal air sac with 0.1 ml. of a broth culture of *E. coli* alone or mixed with 0.1 ml. of the PPLO strain in egg yolk. In a few instances, birds were inoculated in the air sac with PPLO and later exposed to an aerosol of *E. coli*. Birds were killed six, 12, 24, and 48 hours (or sometimes even later) after inoculation. Tissues were fixed in Bouin's fluid and sections were stained with hematoxylin-eosin. At the same time, cultures were made of the air sacs, blood, pericardial sac, and trachea, and the isolated coliforms were identified by means of a plate-agglutination test.

## RESULTS

The experimentally produced lesions were indistinguishable from those of field specimens although the microscopic lesions of the respiratory tracts differed somewhat with the methods of inoculation. It was not possible to differentiate birds inoculated with *E. coli* alone from those inoculated with both *E. coli* and PPLO on the basis of gross or microscopic lesions in any of the organs except the air sacs.

*Air Sacs.*—The air sacs were involved more frequently than any other organ. Many birds from infected flocks were found to have lesions only in the air sacs. In birds inoculated in the air sacs, these sacs were first edematous and about 1 mm. thick (185 times normal thickness). Within 24 hours, the thickness was reduced to 40 times normal and the air sacs were opaque and yellowish with a fibrinous lining. Histologically, the reaction of the experimentally inoculated birds depended on whether they had recived *E. coli* alone or in combination with PPLO. The air sacs of birds receiving *E. coli* alone contained more heterophilic than mononuclear phagocytes while those receiving both agents tended to contain more mononuclear than heterophilic phagocytes.

From the University of Minnesota, Minneapolis. Submitted to the graduate faculty in partial fulfillment of the requirements for the degree of Ph.D. in 1956.

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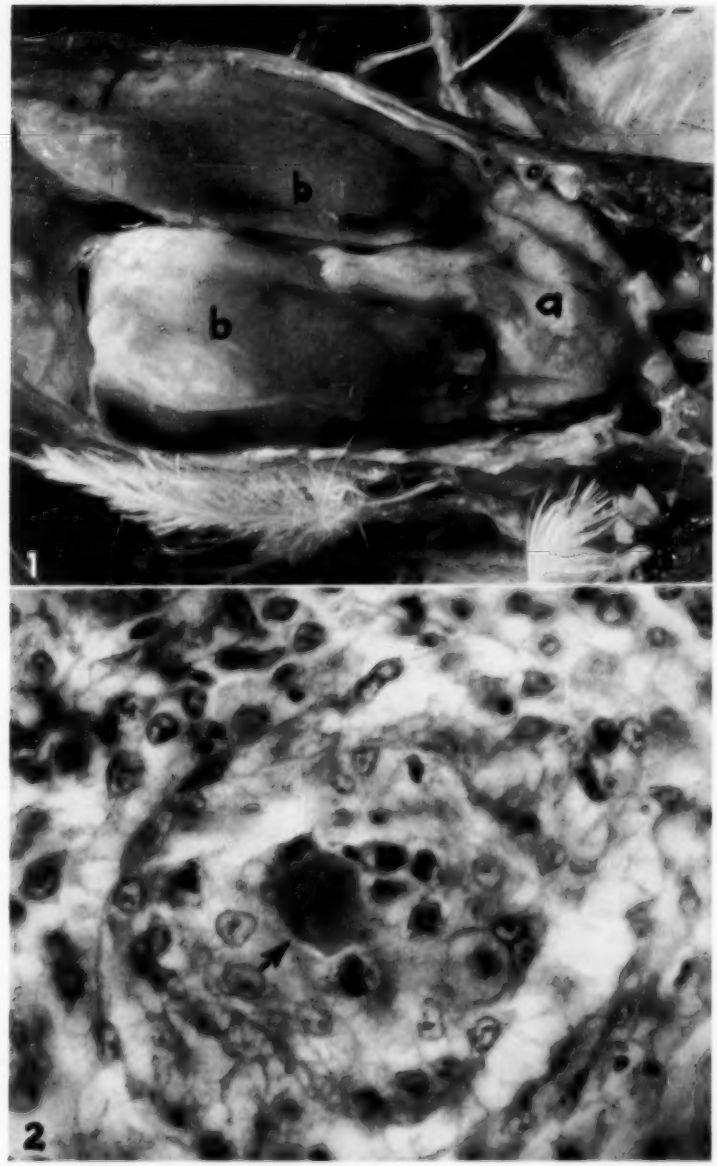



Fig. 1—Fibrinous pericarditis (a) and perihepatitis (b) in a chicken; natural size.

Fig. 2—An early stage of the development of a granuloma in the lung. A mass of bacteria (arrow) is surrounded by heterophils and mononuclear phagocytes. This and all subsequent sections were stained with hematoxylin and eosin; x 1,200.

The connective tissue bundles in air sacs of birds receiving both agents tended to be enlarged and swollen. After 24 hours, mononuclear phagocytes became more prominent in both groups and there were often necrotic areas lined with giant cells. The exudate on the respiratory surface was composed of fibrin, large numbers of necrotic heterophils, a few mononuclear phagocytes, and many bacteria. Later, the surface exudate was often lined with giant cells and mononuclear phagocytes. As infection receded, fibroblasts became more common. Perivascular lymphoid follicles, which are characteristic of PPLO infection,[2,6,8,9] developed after about seven days if the birds had received both PPLO and *E. coli* in the inoculum. The air sacs of birds naturally infected with both PPLO and *E. coli*, as well as those exposed to *E. coli* aerosols following PPLO infection, contained varying proportions of heterophilic and mononuclear phagocytes, as well as remnants of the lymphoid follicles and hyperplastic epithelium of PPLO infection.

*Lungs.*—Gross lesions of the lungs were uncommon in naturally infected birds. Lesions were present in the posteroventral portions of the lungs of about 15 per cent of the air sac-inoculated birds. India ink was similarly deposited in the lung following injection into the air sac. Histologically, the lesions in naturally infected birds often were characteristic of PPLO infection. At times, there was infiltration of the intralobular spaces with mononuclear phagocytes and a few heterophils as well as edema of the tertiary bronchi. Granulomas lined with giant cells or mononuclear phagocytes were sometimes observed in the central portion of the lobules or near the PPLO lesions. Lesions in birds experimentally infected with both agents consisted of infiltration of the lobules and interlobular spaces with varying proportions of mononuclear and heterophilic phagocytes. The infiltration often obscured the normal architecture of the lobules. Necrotic areas, which sometimes contained masses of bacteria, tended to be formed in the central portions of the lobules. These necrotic areas were first lined with heterophils, later with mononuclear phagocytes, and, finally, with giant cells. Healing was by scar formation which sometimes resulted in the destruction of large areas of lung tissue.

*Trachea.*—No lesions were seen in birds experimentally infected with *E. coli* even though *E. coli* could often be isolated from the mucosa. When the birds were also infected with PPLO, there was sometimes thickening of the mucosa. Microscopically, when the birds were also infected with PPLO, there was lymphoid infiltration of the mucosa characteristic of PPLO infection.

*Heart.*—Fibrinous pericarditis was the most striking lesion in the birds and was found in all birds with fatal cases after the first day of infection. The pericardial sac usually showed evidence of infection 12 to 24 hours after air sac inoculation with *E. coli* alone or combined with PPLO. It contained a yellowish, fibrinous exudate with large numbers of coliform bacteria. Adhesions between the pericardium and the epicardium were common, particularly in the later stages of infection. Microscopically, the inflammation was restricted to the pericardium, pericardial sac, and the superficial layers of the myocardium. The first signs of infection consisted of edema and the appearance of a few mononuclear phagocytes in the epicardium. As infection progressed, many more mononuclear phagocytes invaded the area while smaller numbers of heterophilic leukocytes were present. Bacteria, fibrin, and many necrotic heterophils were often present in the pericardial sac. Necrotic areas, which sometimes contained masses of bacteria, were lined with mononuclear phagocytes and, later, with giant cells. Bacteria were often undetectable after four days. Healing, manifested by scars on the epicardium and adhesions to the pericardium, usually occurred within two weeks. It was not possible to differentiate the heart lesions of birds inoculated with *E. coli* alone from those inoculated with both PPLO and *E. coli*.

*Liver.*—A yellowish fibrinous covering 0.5 to 2.0 mm. thick was found on the surface of the livers of many of the birds having pericarditis. Only rarely did perihepatitis occur in birds without gross pericarditis. At times, opaque yellowish areas were found on the surface adjacent to infected air sacs. In a few instances, there were small necrotic areas throughout the liver. Microscopically, the membrane consisted of fibrin, fibroblasts, and a few mononuclear phagocytes. In some cases, there were groups of giant cells in the exudate. In early acute infections, there were a few heterophilic leukocytes in the sinuses, and the Kupffer cells were somewhat enlarged. There was only moderate congestion. It

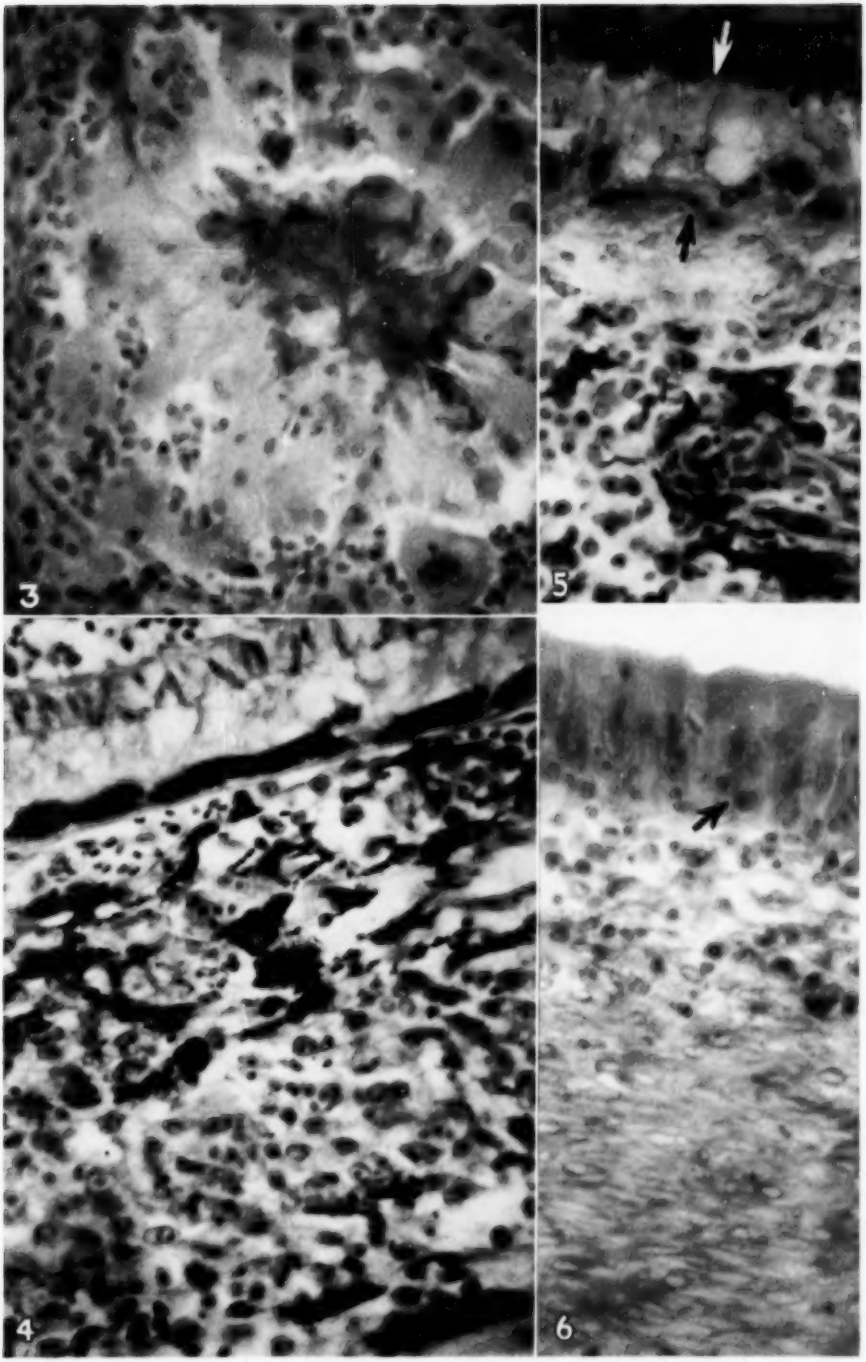



Fig. 3—A late stage in the development of a granuloma of the lung of a chicken; x 1,200.

Fig. 4—An early lesion of the choroid; degeneration of the retina; infiltration by heterophils and mononuclear phagocytes; x 600.

Fig. 5—A later lesion of the choroid. Giant cell reaction (arrows) around a necrotic mass; x 600.

Fig. 6—Salpingitis; infiltration with heterophils and mononuclear phagocytes. Some heterophils (arrow) are passing through the epithelium; x 600.

was not possible to differentiate the liver lesions of the birds inoculated with *E. coli* alone from those inoculated with both PPLO and *E. coli*.

*Oviducts*.—The oviduct was frequently involved following infection of the adjacent left greater abdominal air sac. In these cases, the oviducts were greatly distended and contained a large amount of cheesy, yellowish, fibrinous exudate which was usually free in the lumen. The wall was relatively thin and infection tended to persist for long periods. Microscopically, a few mononuclear phagocytes and fibroblasts were associated with moderate thickening of the wall. Many heterophils could be observed passing into the lumen through the epithelium, which was usually intact. The exudate in the lumen consisted of fibrin, large numbers of necrotic heterophils, and in some cases, masses of bacteria.

*Eye*.—A panophthalmitis was found in about 2 per cent of the experimentally inoculated birds as well as in a small percentage of field cases. The eye infection has been reported previously.[5] Grossly, there was a hypopyon characterized by a greyish deposit in the anterior chamber. Fibrin was deposited and the exudate soon obscured the pupil. Microscopically, the anterior chamber contained heterophilic and mononuclear phagocytes, in varying proportions, as well as fibrin. Usually, the cornea was not involved although, in late stages, there was some vascularization adjacent to the ciliary body. There was edema and mononuclear infiltration of the iris. The ciliary body was infiltrated with heterophilic and mononuclear phagocytes. The choroid was greatly thickened, infiltrated with heterophilic and mononuclear phagocytes, and was often hyperemic. There were often granulomas in the choroid, ciliary body, and the anterior and posterior chambers. The eye lesions were similar whether birds were inoculated with *E. coli* alone or with both PPLO and *E. coli*.

*Body Cavity*.—A peritonitis, with or without pericarditis, which was characterized by the presence of an exudate having the consistency of partially dried egg yolk, throughout the body cavity, was sometimes seen in field birds. The same strains of *E. coli* which are associated with pericarditis may be found in some adult chickens with peritonitis. In field birds, peritonitis was often found without evidence of any other infection.

*Spleen*.—The spleen was of about normal size and at times there was a fibrinous covering on the serous surface.

*Kidneys*.—The kidneys were slightly congested and, in a few instances, had a fibrinous covering on the serous surface. Microscopically, there was little change. In one instance, however, there were necrotic areas involving several adjacent tubules.

*Alimentary Canal*.—Little change was observed but, in a few instances, catarrhal enteritis was present. In acute infections, petechial hemorrhages were sometimes seen on the serous surfaces, particularly of the proventriculus and gizzard.

*Nervous System*.—No gross or microscopic lesions were seen in three specimens of brain, spinal cord, and peripheral nerves which were examined.

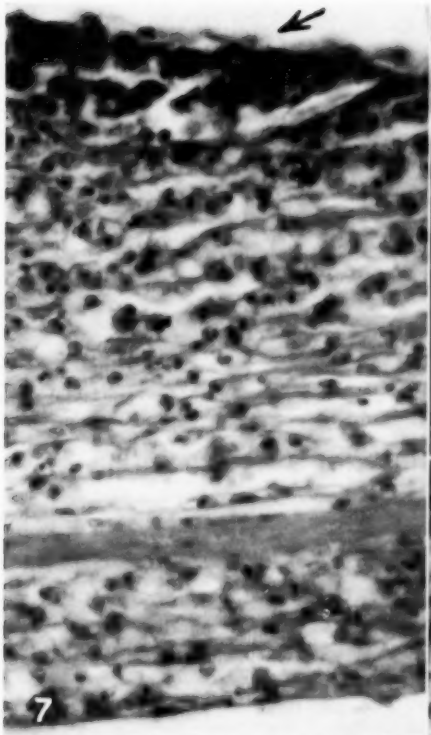
## DISCUSSION

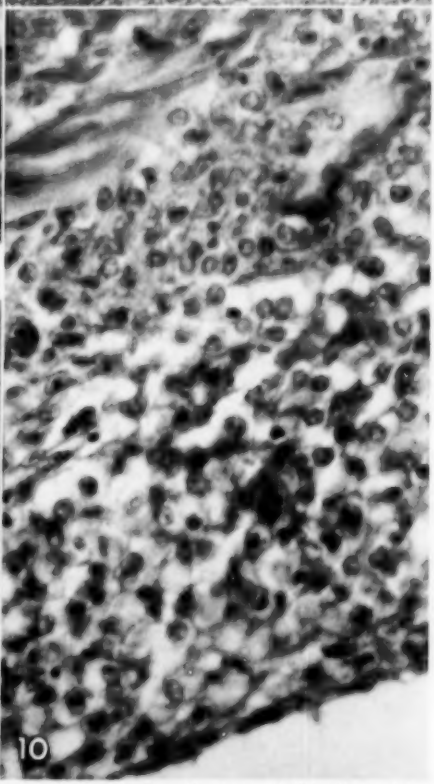
Pericarditis and perihepatitis caused by *E. coli* are almost always associated with concurrent PPLO infection. A pericarditis is also seen in ornithosis of turkeys. *Escherichia coli* infection may be differentially diagnosed by culturing *E. coli* from the pericardial sac since, in ornithosis, the pericardial sac is bacteria-free. Other diseases which sometimes result in pericarditis may be diagnosed by identifying the causative agent. At times, pericarditis produced by *E. coli* may be found without serological, cultural, or histological evidence of PPLO infection.

The pathological picture of infectious serositis of ducks has been described by Daugherty *et al*.[3] The gross lesions appeared similar to those produced by *E. coli* infection. The cellular exudate also consisted of mononuclear and heterophilic phagocytes. Tendency toward formation of granulomas lined with giant cells was not observed in infectious serositis. The central nervous system lesions and signs of infectious serositis were not seen in *E. coli* infection.

Apparently *E. coli* alone[6] does not easily invade the respiratory tracts of normal birds. Infection with some other agent such as PPLO is necessary for certain strains to produce bacteremia, pericarditis, and perihepatitis.

The lesions outside of the respiratory tract, except the salpingitis, probably result from bacteremia. Salpingitis could be produced only following infection of the





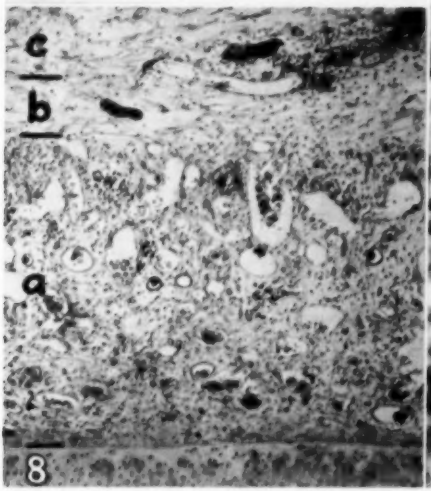



Fig. 7—Air sac of a chicken 24 hours after inoculation with E. coli alone. Infiltration with heterophils and mononuclear phagocytes with fibrin on the respiratory surface (arrow); x 600.

Fig. 8—Healing lesion of choroid (a), retina (b), and vitreous body (c). Masses of mononuclear phagocytes and fibroblasts are between the vessels; x 100.

Fig. 9—Fibrinous pericarditis. Inflammation of pericardium and epicardium; x 100.

Fig. 10—Inflammation of epicardium. Infiltration by mononuclear phagocytes and heterophils; x 600.

adjacent left greater abdominal air sac and was probably the result of direct extension from the air sac. The relatively large number of mononuclear phagocytes in air sacs of birds inoculated with both agents might reflect a stronger defense against a greater number of bacteria. Nakahara [13] found that a strong macrophage reaction in mice resulted in quicker destruction of *E. coli*.

In Hjarre's disease [7, 14] of chickens, which is a chronic disease due to *E. coli*, the necrotic areas of the liver and intestine are lined with giant cells and macrophages. This disease is more chronic than that described in this paper and is not characterized by pericarditis and aerosacculitis.

## Summary

Following several routes of inoculation, certain strains of *Escherichia coli* produced aerosacculitis, fibrinous pericarditis, perihepatitis, salpingitis, and panophthalmitis in chickens. Natural infection was almost always in combination with chronic respiratory disease which is due to a pleuropneumonia-like organism. The cellular infiltration consisted of heterophilic and mononuclear phagocytes in varying proportions. Necrotic areas were, at first, lined with mononuclear phagocytes and, later, by giant cells.

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# Pathogenesis of Arthritis in Chicken Embryos Caused by a Pleuropneumonia-like Organism

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ORGANISMS of the pleuropneumonia group have been shown to cause arthritis in goats, pigs, mice, sheep, cattle, and chickens and have also been associated with arthritis in man.[1,3,8-10,13] There have been descriptions of joint involvement of chicken embryos produced by several strains of avian pleuropneumonia-like organisms (PPLO). In 1948, Delaplane[5] observed purulent arthritis in embryos following inoculation of an agent reported as a chronic respiratory disease virus. Van Roekel, Olesiuk, and Peck[11] described abscesses of the joints due to PPLO. Chute and Cole[2] reported the gross and microscopic changes of an experimental infection in chicken embryos caused by PPLO and included a brief description of the arthritic lesions. It is the purpose of this paper to describe the pathogenesis of arthritis in chicken embryos caused by PPLO.

## MATERIALS AND METHODS

An eighth chicken embryo passage of PPLO was inoculated into the yolk sacs of 48, 8-day-old chicken embryos according to the method of Cunningham.[4] In addition, 20 embryonating chicken eggs were retained as uninoculated controls. Each day, 5 embryos were taken from the inoculated group and 2 from the uninoculated controls and cultured for PPLO on bacto-PPLO agar enriched with 10 per cent horse serum and 1 per cent buffered yeast autolysate (pH 7.8). The PPLO strain used was originally isolated from the pericardial sac of a chicken. This strain was not antigenically related to PPLO of avian origin previously described in the literature.[6,12] Furthermore, it did not utilize sugars according to the fermentation characteristics attributed to this group.[7] The tibiofemoral joints from infected and control embryos were fixed in 10 per cent formalin and stained routinely with hematoxylin and eosin and with Giemsa's stain.

## RESULTS

Of the embryos inoculated with PPLO, 25 per cent were found dead. The pattern of embryo death is shown (table 1). Gross joint involvement was first visible in 1 of 5 embryos on the fifth day after inoculation. On the sixth and subsequent days, all dead embryos exhibited gross joint lesions. The majority of embryos that died between the fourth and eighth day of incubation had extensive cutaneous hemorrhages over the pectoral region. PPLO were present in large numbers in the killed or dead embryos in any of the stages described below.

**TABLE 1—Mortality Pattern Following Inoculation of 48 Embryonating Chicken Eggs, via Yolk Sac, w[illegible] a Pleuropneumonia-like Organism**

| Days after inoculation | No. of deaths | No. of embryos killed | No. showing gross joint lesions |
|---|---|---|---|
| 1 | 0 | 5 | 0 |
| 2 | 0 | 5 | 0 |
| 3 | 1 | 4 | 0 |
| 4 | 8 | 0 | 0 |
| 5 | 5 | 0 | 1 |
| 6 | 3 | 2 | 3 |
| 7 | 3 | 2 | 5 |
| 8 | 2 | 7 | 9 |

*Pathological Findings.*—The tibiofemoral articulations of embryos infected with PPLO were enlarged and pale (fig. 1).

Microscopically, the joint lesions developed in five stages. Stage one was observed on the first and second days after inoculation. The joints showed hyperemia

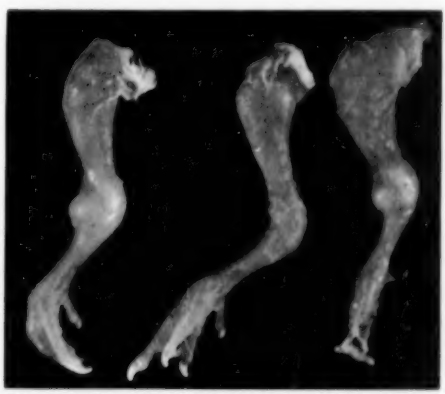

**Fig. 1—Enlarged tibiofemoral joints of 3 inoculated chicken embryos.**

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Fig. 2—Stage one in the development of the arthritic lesion. This tibiofemoral articulation is normal except for beginning slough of synovial cells in the synovial cavity to the left; x 80.

and necrosis of the synovial cell layer in the region of the synovial recesses (proximal and distal areas of the joint cavity where the synovial membrane joins the articular cartilage, fig. 2). In stage two, seen on the third day, increasing numbers of mononuclear cells accumulated in hyperemic blood vessels and infiltrated the joint capsule in the region of the proximal and distal reflections. Necrosis of the synovial layer became more severe. Stage three appeared on the fourth and fifth days. It was characterized by greater numbers of mononuclear cells in local blood vessels and the synovial layer. The joint cavity remained free of inflammatory cells. Necro-

Fig. 3—Stage four, showing necrosis of the articular cartilage. Mononuclear cells are present in the joint cavity; x 80.

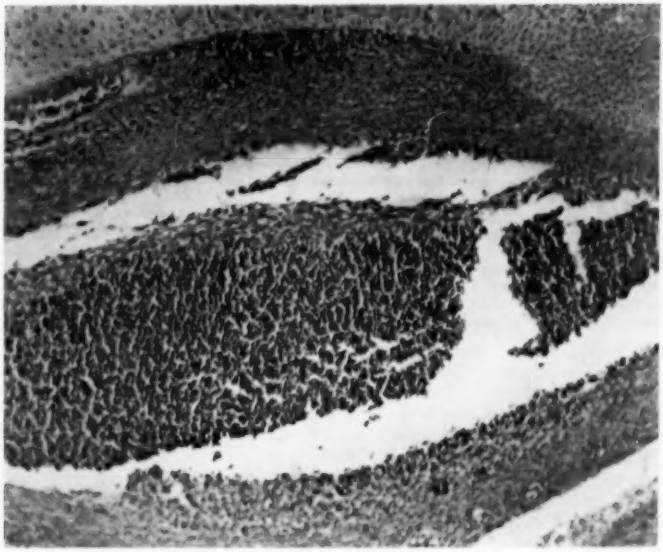

Fig. 4—Stage five, fibrous thickening of the joint capsule (below) and fibrous replacement of necrotic articular cartilage (above). Mononuclear cells are present in the joint cavity; x 80.

sis of the synovial cell layer extended into the superficial layers of the articular cartilage. At first, the surface of the articular cartilage appeared loose and frayed but, later, frank necrosis extended four or five cell layers deep. On the sixth and seventh days after inoculation, stage four was observed. At this time, mononuclear cells invaded the joint cavity, especially the proximal and distal recesses. During the latter part of this stage, many of the infiltrating cells became necrotic. Necrosis of the joint capsule and articular cartilage became more severe during this stage (fig. 3). The blood vessels contained fewer erythrocytes and more mononuclear cells than normally. Stage five was seen on the eighth day after inoculation. Hyperemia was less pronounced and fewer mononuclear cells were found within the blood vessels. In this organizational stage, the joint capsule showed fibrous thickening and the joint cavity was engorged with dead and dying mononuclear cells (fig. 4). Connective tissue proliferation (pannus) replaced necrotic articular cartilage and formed adhesions between proximal and distal bones. Foci of mononuclear cells in periarticular sites were encapsulated by connective tissue and, in a few cases, surrounded by foreign body granulomatous tissue.

Articulations of uninoculated control embryos exhibited no gross or microscopic lesions at any stage.

## Discussion and Summary

Examination of chicken embryos, selected one to eight days after inoculation with PPLO, revealed the development of arthritic lesions from the earliest stage of hyperemia to the stage of fibrous organization. Necrosis began early in the process and involved first the synovial cell layer and, later, the articular cartilage. Necrosis preceded cellular invasion of the joint cavity. The joint capsule was infiltrated by mononuclear cells by the third day, but the joint cavity was free of cells until the sixth day. From the sixth day on, mononuclear cells accumulated in great numbers in the joint cavity. The vascular and cellular components of the inflammation subsided by the eighth day when the process became organized.

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# Infectious Synovitis Control by Medication — Effect of Strain Differences and Pleuropneumonia-like Organisms

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INFECTIOUS SYNOVITIS (IS) has appeared as an important disease in broiler-producing areas of the United States.[4,6,8,11,17] Mortality has usually been low, for the course of the disease is generally prolonged. The joints become swollen and sore, which impairs the mobility of the bird. Feed utilization becomes less efficient. As the mobility is restricted, less feed and water are consumed until life is maintained at the expense of body tissues. The morbidity has ranged from 2 to 75 per cent and mortality up to 20 per cent of the flock. Emaciation and the purulent or caseous exudates in the synovial or bursal sacs have resulted in "trim outs" and condemnations of infected birds during postmortem inspection.[4]

The disease has been controlled, under laboratory conditions, by the use of antibiotics. Flock treatment had proved worthwhile since it prevents the disease from spreading and because birds in early stages of the disease usually show improvement. However, because of complicating diseases, treatment of affected birds in field flocks has been only partially successful.

This is a report of laboratory studies using chlortetracycline and furazolidone (NF-180) on the disease produced by different agents isolated from birds with infectious synovitis or synovitis due to pleuropneumonia-like organisms (PPLO).

## REVIEW OF LITERATURE

Chlortetracycline and oxytetracycline have had the greatest therapeutic value for the control of infectious synovitis in chickens. When fed continuously at 200 Gm. per ton of feed, no signs, except for temporary swellings, developed during a ten-week observation period among laboratory birds experimentally exposed to infectious synovitis. The infection developed in 2 birds treated with oxytetracycline following withdrawal of the drug from the feed at 7 weeks of age. Chlortetracycline was more effective than oxytetracycline in causing a remission of swellings.[12-15] Intermittent treatment with chlotetracycline was effective during the periods of medication.[9,15]

Continuous feeding of 100, 200, or 300 Gm. of chlortetracycline per ton of feed, starting 19 and two days before inoculation, on the day of inoculation, and two and four days after inoculation, was effective in controlling synovitis. When started eight and 12 days after inoculation, the medication was only partially effective. The amount of medication necessary to prevent the disease was lower than that needed for treatment.[9] Levels lower than 100 Gm. have had some therapeutic value.[12,16] Tetracycline has shown slight value against synovitis.[16]

Furazolidone (200 Gm./ton) was ineffective in controlling synovitis in birds exposed intravenously and intratracheally.[12] In birds inoculated in the foot pad, it was only slightly effective.[16]

Streptomycin sulfate, penicillin, bacitracin, para-aminobenzoic acid, triple sulfonamides (Whitmoyer), erythromycin, magnamycin, chloromycetin, furadantin (NF-153), sulfaquinoxaline, and sulfamethazine were ineffective in controlling synovitis.[12-16] Oxytetracycline, in oil suspensions injected subcutaneously, delayed the development of lesions.[14,16] Streptomycin was partially effective when injected at the time of inoculation but not after signs developed.[9]

It has been reported that one strain of the infectious synovitis organism, previously treated with streptomycin, oxytetracycline, or furazolidone and injected into embryos, was resistant to these drugs, yet tetracycline reduced the embryo mortality.[9] *In vivo* studies with other isolates have shown tetracycline to be less effective than oxytetracycline when given in the feed.[16]

## MATERIALS AND METHODS

The infective agents of IS were isolated from flocks in which the clinical diagnosis was IS, chronic respiratory disease (CRD), bluecomb, hemorrhagic disease, or an undiagnosed respiratory infection (table 1). The birds from which the agents were isolated had a purulent exudate in one or more joints and their condition could have been diagnosed as infectious synovitis.

All isolations had been made by inoculation of the selected material into the yolk sac of embryonating eggs and had been in storage at −20 C. for two to 12 months. To obtain a uniform pathogenicity, embryo yolk cultures of each isolate were

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**TABLE 1—Clinical History of Poultry Flocks from Which IS and PPLO Agents Were Isolated**

| Culture (No.) | Source of isolated agent | Clinical flock diagnosis | Mortality (%) | Morbidity (est.%) |
|---|---|---|---|---|
| 1 | Hock | Synovitis | Unknown | Unknown |
| 2 | Hock | Synovitis | 4.4 | 5.0 |
| 3 | Spleen | Synovitis | 3.2 | 0.5 |
| 4 | Hock | Bluecomb | 1.7 | 0 |
| 5 | Hock | Mixed infection* | 10.0 | 75.0 |
| 6 | Hock | Synovitis | 2.0 | 2.0 |
| 7 | Hock | Mixed infection* | 10.0 | 75.0 |
| 8 | Hock | Synovitis | 1.0 | 5.0 |
| 9 | Mucus | Mixed infection* | 10.0 | 75.0 |
| 10 | Hock | CRD | 1.3 | 0 |
| 11 | Broth culture of PPLO from culture 10 | | | |

* Infectious synovitis, hemorrhagic syndrome, and an undiagnosed respiratory infection. Isolations from the same flock.

thawed and another embryo passage was made. Then each isolate was inoculated into 8 chicks via the foot pad. Seven days later, these chicks were killed and a broth suspension was prepared from the synovial fluid, caseous exudate, or the granulomatous-like thickening of the inoculated foot pad. The suspension was injected into the yolk sac of 5-day embryonating eggs. Three consecutive embryo passages of each isolate were made and the third was used for chicken inoculation in experiments 1 and 2. Embryonating eggs used for the third embryo passage were inoculated so that the isolates used for chicken inoculation were held at −20 C. for a maximum of six days.

The broth suspension of each isolate, as prepared above, was cultured in PPLO broth (Difco) with 10 per cent horse serum and 10 per cent yeast hydroylsate, nutrient agar, and thioglycollate medium. PPLO were recovered from one isolate (culture 10). A nine-day broth culture was prepared for chicken inoculation and designated culture 11.

## EXPERIMENTAL PROCEDURE

*Experiment 1.*—Day-old New Hampshire chicks (480), obtained from the University flock, were randomly distributed, 12 birds per battery, in 40 electrically heated batteries. All were fed the standard chick and broiler ration suggested by Briggs *et al.*,[2] which contained 4 Gm. of procaine penicillin per ton. Two batteries of chicks were maintained as unexposed control birds. Normal egg yolk was injected into the foot pads of birds in one of these batteries. The other 456 chicks were infected by injecting each in the foot pad with 0.25 ml. of infective yolk from the third embryo passage of one of the ten agents isolated from birds with IS and of PPLO, culture 10 (table 1), or with 0.25 ml. of the PPLO broth (culture 11). One battery of birds infected with each agent was used as an infected control or was fed, from the first day, on the basal ration containing 100 to 200 Gm. of crystalline chlortetracycline hydrochloride* per ton. Other batteries of chicks, inoculated with IS isolates 7 through 9 and PPLO cultures 10 and 11, were fed the basal ration containing 200 Gm. of NF-180† per ton (table 2). The birds were observed daily.

* Supplied by Lederle Laboratories Division of American Cyanamid Company, Pearl River, N. Y.

† Supplied by Hess and Clark, Inc., Ashland, Ohio.

**TABLE 2—The Influence of Infectious Synovitis and PPLO on Weight Gain (in 41 Days); Degree of Swelling at the Site of Injection; Extension of Infection to Other Joints; and Mortality in Nonmedicated Infected Chicks and in Chicks Given Chlortetracycline or Furazolidone**

| Treatment | Infectious synovitis isolates | | | | | | | | | PPLO cultures | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| Controls,* infected | | | | | | | | | | | |
| Weight gain (Gm.) | Dead | Dead | Dead | 383 | Dead | Dead | 503 | 37 | 14 | 283 | 309 |
| Degree of swelling | 9.0 | 9.0 | 9.0 | 1.9 | 9.0 | 9.0 | 1.3 | 9.0 | 9.0 | 8.2 | 7.4 |
| No. with extended infec. | 10† | 12 | 9 | 0 | 10 | 12 | 0 | 11 | 10 | 6 | 4 |
| No. died | 12† | 12 | 12 | 0 | 12 | 12 | 0 | 11 | 11 | 0 | 0 |
| Chlortetracycline (100 Gm./ton) | | | | | | | | | | | |
| Weight gain (Gm.) | 488 | 511 | 504 | 404 | 490 | 557 | 438 | 456 | 467 | 409 | 441 |
| Degree of swelling | 0 | 0.2 | 0 | 3.4 | 0.2 | 0 | 1.1 | 0.2 | 0 | 5.2 | 6.9 |
| No. with extended infec. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| No. died | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Chlortetracycline (200 Gm./ton) | | | | | | | | | | | |
| Weight gain (Gm.) | 461 | 442 | 528 | 380 | 517 | 433 | 429 | 531 | 453 | 409 | 442 |
| Degree of swelling | 0 | 0.1 | 0 | 2.7 | 0 | 0 | 1.2 | 0 | 0 | 1.6 | 0.75 |
| No. with extended infec. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| No. died | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Furazolidone (200 Gm./ton) | | | | | | | | | | | |
| Weight gain (Gm.) | ..... | ..... | ..... | ..... | ..... | ..... | 441 | 174 | 133 | 275 | 306 |
| Degree of swelling | ..... | ..... | ..... | ..... | ..... | ..... | 0.8 | 7.6 | 7.4 | 7.9 | 7.8 |
| No. with extended infec. | ..... | ..... | ..... | ..... | ..... | ..... | 0 | 10 | 10 | 3 | 3 |
| No. died | ..... | ..... | ..... | ..... | ..... | ..... | 0 | 5 | 5 | 0 | 0 |

* None of the noninfected controls developed signs of synovitis. Their weight gains were: 12 noninfected birds—471 Gm. and 12 egg yolk-injected birds—479 Gm.; † 12 chicks in each battery.

Twice a week, each chick was weighed and a record made of the degree of swelling of the inoculated leg and of whether the infection had extended to other joints. The degree of swelling was classified,[18] with zero indicating no swelling and numbers 1 to 9 indicating gradations from a slight swelling of the inoculated foot pad to a marked swelling of the pad, tendon sheath, and hock joint. At 41 days of age, medication was removed from all birds, except those exposed to PPLO culture 10, and the effect on the further development of lesions was studied (table 3). All birds exposed to PPLO cultures 10 and 11, and 2 birds from each of the batteries exposed to the other isolates, were serologically tested for CRD antibodies using the standard plate antigen.[1]

*Experiment 2.*—Since isolates 4 and 7 did not produce typical signs of infectious synovitis, the 84 birds previously inoculated with them were divided into groups and challenged with all nine IS isolates and the two PPLO cultures (table 4). Birds of the same age (47 days), that had not been previously inoculated, were challenged with each isolate to serve as controls.

## RESULTS

The degree of pathogenicity of the IS isolates differed for embryos and chickens. Isolates 4 and 7 were most pathogenic for chicken embryos and were least pathogenic for chickens. Isolates 4 and 7 had a median infection time before death of five and three days, respectively, and none of the embryos survived. The pathogenicities of the other IS isolates were similar for chickens and embryos. The median time before death for embryos was eight to ten days; approximately 5 per cent survived. For PPLO culture 10, the median time before death of embryos was six days; none survived.

In general, the results of experiment 1 suggest that there were three different pathogenic agents.

The similarity of the results with isolates 1, 2, 3, 5, 6, 8, and 9 indicated that they were equally pathogenic. Weight gains of untreated chicks inoculated with these isolates were markedly depressed and the degree of swelling in the inoculated leg and the number of birds with an extension of the infection to other joints were about equal, irrespective of the isolate. Mortality was high.

Chlortetracycline was equally effective in controlling the diseases caused by each of these isolates. Furazolidone had only slight therapeutic value against the disease caused by isolates 8 and 9. Withdrawal of chlortetracycline (table 3) resulted in the development of swellings in the inoculated foot pads of 7 birds.

Isolates 4 and 7 produced a similar infection but they differed in pathogenicity from the other infectious synovitis isolates (table 2). The birds inoculated with these isolates gained less but there was no mortality. There was no detectable spread of the infection and the amount of swelling in the inoculated legs rated 3.4 or less. Chlortetracycline had no therapeutic value against either infection and furazolidone had none against the infection caused by isolate 7. Recovery occurred in unmedicated as well as medicated birds (table 3). The broth culture (11) appeared

**TABLE 3—The Development of Infectious Synovitis Following Removal of Medication**

| Isolate used for inoculation | No. of birds showing swellings in the pad or hock joints before and after removal of medication | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | No medication | | Chlortetracycline (100 Gm./ton) | | Chlortetracycline (200 Gm./ton) | | NF-180 (200 Gm./ton) | |
| | Before | After* | Before | After* | Before | After* | Before | After* |
| IS 1 | All dead | | 0 | 1 | 0 | 1 | .... | .... |
| IS 2 | All dead | | 2 | 4 | 0 | 0 | .... | .... |
| IS 3 | All dead | | 0 | 1 | 0 | 0 | .... | .... |
| IS 4 | 10 | 4 | 12 | 9 | 12 | 7 | .... | .... |
| IS 5 | All dead | | 3 | 2 | 1 | 1 | .... | .... |
| IS 6 | All dead | | 0 | 1 | 0 | 0 | .... | .... |
| IS 7 | 10 | 5 | 11 | 4 | 12 | 4 | 9 | 4 |
| IS 8 | All dead | | 1 | 1 | 0 | 0 | 9 | 7§ |
| IS 9 | All dead | | 0 | 0 | 0 | 0 | 9 | 7§ |
| PPLO 10 | 12 | 12 | 11 | 10‡ | 5 | 5 | 12 | 11† |
| PPLO 11 | 12 | 11 | 12 | 10 | 6 | 5 | 12 | 12 |
| Egg yolk | 0 | 0 | .... | .... | .... | .... | .... | .... |
| None | 0 | 0 | .... | .... | .... | .... | .... | .... |

* Observation made eight days after removal of medication, culture 11; ten days, isolates 4 and 7; 13 days, isolates 1–3, 5, 6, 8, and 9. Medication was not removed from birds exposed to culture 10, observation made at ten days. † One bird killed. ‡ One bird died, but not as a result of synovitis. § Five birds died as a result of synovitis.

**TABLE 4—Susceptibility of Birds Previously Exposed to Isolates 4 and 7 when Subsequently Exposed to IS and PPLO Isolates**

| Challenge isolate | Isolate 4 * | Isolate 7 * | Controls * not previously exposed |
|---|---|---|---|
| 1 | 1/ | 4/4 | 5/5 |
| 2 | 2/3 | 4/4 | 5/5 |
| 3 | 3/3 | 4/4 | 5/5 |
| 4 | 0/3 | 0/4 | 0/5† |
| 5 | 3/3 | 4/4 | 5/5 |
| 6 | 3/3 | 4/4 | 5/5 |
| 7 | 0/3 | 0/4 | 0/5‡ |
| 8 | 3/3 | 4/4 | 5/5 |
| 9 | 3/3 | 4/4 | 5/5 |
| 10 | 3/3 | 4/4 | 4/4 |
| 11 | 3/3 | 4/4 | 4/4 |
| None | 0/3 | 0/4 | 0/5 |

* No. affected per No. challenged. † All had a No. 4 swelling four days after inoculation. ‡ One bird had a No. 1 swelling four days after inoculation.

to affect the birds less severely than the embryo yolk culture (10) of the same PPLO as evidenced by lesser weight gains. Neither caused mortality. Against these cultures, chlortetracycline had a definite but limited therapeutic value at 100 Gm. per ton. At 200 Gm. per ton, it was more effective and prevented the appearance of PPLO synovitis in about 58 per cent of the birds. It would seem that the birds had developed increasing resistance to the infection since improvement was noticed after as well as before withdrawal of medication. NF-180 was ineffective against both cultures 10 and 11.

The blood serum from 2 birds exposed to IS isolates 1 to 9 were tested for CRD antibodies and found negative. Serums from all birds exposed to PPLO cultures 10 and 11 were positive, except from those fed 200 Gm. of chlortetracycline, where three serums from birds exposed to culture 10 and five from birds exposed to culture 11 were negative. These tests were conducted 23 days after inoculation.

Immunity tests were made on birds previously inoculated with isolates 4 and 7 (experiment 2). Results (table 4) show that, of these birds, 24 of 27 (isolate 4) and all of 36 (isolate 7) developed signs of synovitis when exposed to the IS isolates or PPLO cultures other than 4 and 7. Neither isolate 4 nor 7 caused swellings to appear in these birds. In birds not previously challenged with isolates 4 and 7, all of 43 developed signs of the infection. Isolates 4 and 7 caused only temporary swellings in birds not previously exposed, so they were essentially nonpathogenic. It appears that isolates 4 and 7 were not immunologically related to the other IS isolates or to PPLO; however, additional trials would be needed to determine whether these observations were significant.

## DISCUSSION

From eight different flocks of chickens, nine isolates of IS and one isolate of PPLO were obtained. A study of these isolates in chicken embryos and in laboratory chickens indicated that three different pathogenic agents were present. These were: (1) a viral agent which, when inoculated into chicks, produced typical signs and lesions of IS; (2) PPLO which, when inoculated into chicks, produced signs of infection clinically similar to those produced by the IS agent; and (3) an agent which was pathogenic for chicken embryos but relatively nonpathogenic for chickens. When inoculated into chickens, the PPLO, whether cultured in embryo or broth, did not cause extreme emaciation, caseous exudate in the synovial and bursal membranes, enlarged and discolored livers, or splenomegaly. Preliminary blood studies have shown that the infectious synovitis and that caused by PPLO could not be differentiated on the basis of blood counts or hemoglobin values.[12]

The improper diagnosis of the etiological agent in clinical cases of synovitis plus the presence of intercurrent diseases could cause the recommendation of relatively ineffective treatment with variable results. For treatment to be of the greatest value, an early diagnosis is imperative. Appropriate concentrations of medication should be determined by the condition of the flock.

The minimum concentration of chlortetracycline which was found to be effective for treatment of infectious synovitis was approximately 200 Gm. per ton of feed.[2,13] Continuous medication was necessary.[2,9,15,18] If the antibiotic was discontinued, the disease frequently recurred.

For prevention of infectious synovitis, lower concentrations of chlortetracycline, given continuously, have been effective.[2,13,16] In laboratory birds, excellent results have been obtained with 100 Gm. of chlortetracycline per ton. However, in controlled field experiments, continuous medication with 50 Gm. has given good clinical control.[10]

Furazolidone (NF-180) has been used alone and in combination with antibiotics in some areas.[5,6] This report and others have shown that NF-180 has essentially no clinical value in the treatment of infectious synovitis.[14,16,18] If given as a preventive medicant, it has only a slight value. The

success of chlortetracycline and the failure of NF-180 for control of PPLO which cause synovitis differs greatly from the results of its use in the *in vitro* studies on PPLO.[7]

## SUMMARY

Nine infective agents were isolated from the joint purulent exudate, tracheal mucus, or spleens of birds with infectious synovitis (IS) from seven flocks. PPLO was isolated from the joint purulent exudate of 1 bird of another flock. When inoculated into the foot pads of chickens, seven of the nine IS isolates produced signs and lesions typical of IS. The other two IS isolates produced only temporary swellings in the inoculated foot pad. The PPLO isolate produced lesions in chickens similar to those produced by IS. PPLO antibodies were found in the serum from chickens previously inoculated with the PPLO isolate but not in that from those inoculated with the nine isolates of IS.

Experiments were conducted to test the susceptibility to the isolated agents of chicks fed chlortetracycline and NF-180. Chlortetracycline was effective, whereas NF-180 was essentially ineffective in the control of infectious synovitis. Neither drug had therapeutic value against the disease caused by the two atypical strains of the IS agent. Chlortetracycline was partially effective, but NF-180 was ineffective against the disease caused by the PPLO agent.

Under the conditions of this experiment, the two atypical strains of the IS agent induced immunity to only the agent in these strains. Similar tests with the other isolates were not possible.

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# Relationship Between Immunity and Serum-Neutralization Titers in Chickens Inoculated with Three Infectious Bronchitis Vaccines

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THE SERUM-NEUTRALIZATION (SN) test has been applied to the study of infectious bronchitis (IB) by several authors,[3-6, 9] and is currently used to determine the immunological status of chickens previously exposed to this virus. Very little information, however, is available concerning the reliability of the SN test as a gauge for measuring the degree of immunity to IB.[4, 5]

The purpose of the investigation reported herein was to determine the relationship between the challenge results and the SN titers of the serums of vaccinated birds.

## MATERIALS AND METHODS

The strains of infectious bronchitis virus (IBV) used in this experiment were commercially available IB vaccines, identified in this study as A, B, and C.

White Leghorn chickens classified as "susceptible" and "commercial" were used. The "susceptible" birds were hatched from hens susceptible to IB and the "commercial" birds came from two hatcheries where the breeding flocks were known to be vaccinated with IBV or had been naturally exposed to the disease.

Each strain of IBV was studied at a different time, and the principals were kept under isolation. The vaccines were reconstituted according to the manufacturer's instructions, and each bird was given 1 drop of the vaccine by either the intranasal or conjunctival route. At an average of 30 days after vaccination, every bird in the experiment was challenged intratracheally with 10,000 $l.d._{50}$ of IBV, Massachusetts strain,* suspended in 0.2 ml. of tryptose broth. Immediately before challenge, individual blood samples were obtained from a number of birds selected at random. Chickens that showed moist râles, gurgling, sneezing, and coughing within 12 days after challenge were considered either susceptible or not completely resistant. Absence of these respiratory signs, within the same period of time, indicated resistance. At the time of challenge, 4 susceptible chickens were also inoculated intratracheally with the Massachusetts strain of IBV and served as controls.

The procedure followed in the SN test was similar to that used for Newcastle disease[2] and IB[5, 7] with slight modifications.[1] The virus-dilution method was used. The serums were diluted 1:10 in sterile physiological saline solution and inactivated at 56 C. for 30 minutes. The dilution range of the IBV* used was $10^{-2}$ to $10^{-6}$ because preliminary titrations of this virus indicated that the $l.d._{50}$ was in the range of $10^{-5}$ dilution. All virus dilutions were held in ice water until inoculation. The chicken eggs were candled twice daily and observed for six days. Eggs found to contain dead embryos within 24 hours after inoculation were discarded, and the mortality was considered nonspecific because previous trials indicated that the earliest deaths caused by this strain of IBV occurred at approximately the fortieth hour. A SN titer of $10^2$ or higher was considered evidence of a specific response to IBV. A SN titer of $10^1$ or less was interpreted as insignificant and nonspecific.

## RESULTS

The results are tabulated (table 1).

*Response to Strain A.*—The neutralization titers following vaccination with this strain were determined in three age groups of chickens—1 day, 3 days, and 5 weeks. A direct relationship was observed between a satisfactory degree of immunity to challenge 27 days after vaccination and the SN titer in the serums of the same birds. The origins of the three groups of birds tested did not seem to influence the responses of the birds to challenge (98 and 100% of immunity) or their SN response to the vaccine ($10^4$ or greater neutralizing doses in the serums of all age groups). The SN titers of birds vaccinated by instilling the virus suspension into the conjunctival sac were similar to those obtained in the birds vaccinated intranasally.

*Response to Strain B.*—A direct relationship was observed between significant SN titers and resistance to challenge for the majority (93.2%) of the serums tested

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* Obtained through the courtesy of Dr. F. S. Markham of the American Cyanamid Co., Pearl River, N. Y.

**TABLE 1—Response of Birds to Three Commercial Vaccine Strains of Infectious Bronchitis Virus (IBV)**

| IBV strain | Experimental chickens: No. of birds | Age | Hatching source | Route of inoculation | Immune * (%) | No. of serum samples from randomly selected birds | SN titer | Discrepancies between SN titer and challenge results (%) |
|---|---|---|---|---|---|---|---|---|
| A | 40 | 1 day | Commercial | Conjunctival | 98 | 40 | 10 — $10^4$ or higher | 0 |
| | 40 | 3 days | Susceptible | Conjunctival | 98 | | 10 — $10^4$ or higher | 0 |
| | 40 | 3 days | Susceptible | Intranasal | 100 | | 10 — $10^4$ or higher | 0 |
| | 25 | 5 weeks | Susceptible | Intranasal | 100 | | 10 — $10^4$ or higher | 0 |
| B | 50 | 3 days | Susceptible | Intranasal | 98 | 44 | 3 — $10^1$<br>14 — $10^2$<br>27 — $10^3$ or higher | 6.8 † |
| | 50 | 5 weeks | Commercial | Intranasal | 94 | 38 | 36 — $10^3$ or higher<br>2 — $10^4$ or higher | 7.9 ‡ |
| C | 34 | 2 months | Susceptible | Intranasal | 100 | 32 | 1 — $10^1$<br>9 — $10^2$<br>12 — $10^3$<br>9 — $10^4$<br>1 — $10^5$ | 3.1 † |
| | 46 | 6 days | Commercial | Intranasal | 41 | 41 | 1 — $10^1$<br>18 — $10^2$<br>22 — $10^3$ | 14.6 — 2.4 † ; 12.2 § |

* On the basis of intratracheal challenge with 10,000 l.d.$_{50}$ IBV, Massachusetts strain. Strain A was challenged on the twenty-seventh, strain B on the thirtieth, and strain C on the thirty-third day after vaccination. † Percentage of birds showing $10^1$ IBV neutralization doses but resistant to challenge. ‡ Percentage of birds showing a significant SN titer ($10^2$ or higher) but not completely resistant to challenge. § Percentage of birds showing a significant SN titer ($10^2$ or higher) but susceptible to challenge.

in the 3-day-old chicks. However, a few birds (6.8%) were resistant to challenge 30 days after vaccination, although their serums showed a SN titer of $10^1$.

It was also of interest that 7.9 per cent of the 5-week-old birds were not completely resistant to the challenge dose of IBV. The serums, however, contained $10^3$ to $10^4$ or more IBV neutralizing doses, amounts which are significant and representative of immunity.

*Response to Strain C.*—A direct correlation appeared between the SN response and the behavior of the birds toward challenge in the majority (96.9%) of the 2-month-old birds (table 1). Only 3.1 per cent of the birds with insignificant SN titers ($10^1$) were found resistant to challenge 33 days after vaccination.

The serological response of the 6-day-old "commercial" chicks at the time of challenge indicated that 85.4 per cent of the birds behaved as expected, but 14.6 per cent showed either a significant SN titer with no corresponding resistance to challenge (12.2%) or an insignificant SN titer with an unexpected resistance to challenge (2.4%).

## Discussion

It is generally believed that significant SN titers ($10^2$ or higher) for IBV in vaccinated birds represents immunity to the virus. The results of studies comparing SN titers, approximately one month after vaccination, with three commercial strains of IBV, with concurrent challenge data, show that such belief is supported by serological and immune responses of birds vaccinated with strain A. A few discrepancies between SN titers and challenge results were noticed with strain B (table 1).

In the case of strain C, however, marked discrepancies were observed in the 6-day-old chicks (14.6%) among which the SN titers did not parallel the expected challenge response.

These results indicated that with strains B and C neither the SN test nor intratracheal challenge seemed to provide absolute means of detecting an immune status of birds against IB. Further work is being undertaken to determine the frequency and significance of these discrepancies.

## Summary

A study of the relationship between serum-neutralization (SN) titer and response to intratracheal challenge of birds vaccinated with three strains of infectious bronchitis virus (IBV) showed that a direct correlation between them existed in only one of the three vaccines tested.

Noticeable discrepancies between the SN

titers and the challenge results were observed in birds vaccinated with the other two strains of IBV.

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# Cultivation and Cytopathogenicity of Newcastle Disease Virus in Hela and Bovine Kidney Cell Cultures

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NEWCASTLE DISEASE virus (NDV) can be readily propagated in chicken embryonating eggs and *in vitro*, using fragments of chicken embryo tissues suspended in a suitable liquid medium.[1,3-5,8] However, since fertile chicken eggs known to be free of infections are becoming increasingly difficult to obtain, cultivation of the virus on tissues other than those of avian origin has a definite advantage.

This report describes a method of cultivating two strains of Newcastle disease virus, of divergent virulence for chickens, in monolayer cultures of Hela and bovine kidney cells.

## MATERIALS AND METHODS

*Tissue Culture Medium.*—A modified Earle's salt solution containing 0.5 per cent lactalbumin hydrolysates and 0.1 per cent yeast extract was used to suspend, propagate, and maintain the bovine fetal kidney and Hela cell growth.[7] To the medium were added 250 units of penicillin and 0.25 μg. of dihydrostreptomycin per milliliter of fluid. When the nutrient fluid was to be used for fetal kidney cell growth and propagation of the virus, 6 per cent sterile lamb serum was added. Modified Earle's solution containing 10 per cent human serum was used to initiate Hela cell growth during the first 48 to 72 hours. To maintain cell growth, and after the Hela cells were inoculated with the virus, the human serum was replaced with 10 per cent lamb serum.

*Bovine Fetal Kidney Cells.*—The kidney cells were obtained from kidneys of fetuses of cattle brought to a local slaughterhouse in the fifth to eighth months of gestation. The kidneys were removed under sterile conditions and the encapsulated kidneys suspended in Dulbecco's phosphate buffer solution (PBS). Within one hour after removal from the animal, they were minced and trypsinized in 0.25 per cent trypsin in PBS, as described by Dulbecco and Vogt.[6]

The cell suspension was centrifuged at 800 r.p.m. and resuspended in the liquid culture medium to make a final cell dilution of 1:200. The suspension (in 2-ml. amounts) was placed in Pyrex screw-cap test tubes (16 by 125 mm.). Incubation was carried out in stationary racks at 35 C. for 48 to 72 hours. When the pH of the liquid medium dropped to the acid side (6.7 or less), the medium was replaced with the same amount of fresh medium. Usually by the sixth or seventh day, the tubes presented a contiguous sheath of cells and were ready for inoculation.

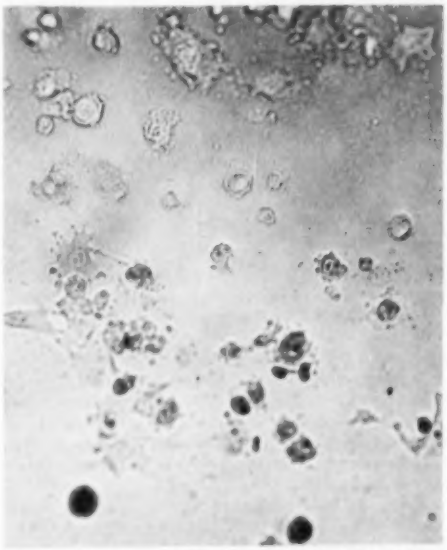

**Fig. 1—Hela cell tissue culture inoculated 48 hours previously with strain GB of NDV, illustrating cytopathogenic effect of virus on cells and destruction of the sheath. Approximately x 133.†**

*Hela Cells.*—Cells were obtained from the 103rd passage level of the x4–5 line of Hela cells.* The culture was maintained in 200-ml. milk dilution bottles. Each was inoculated with approximately 1,000,000 cells suspended in modified Earle's solution containing 10 per cent human serum. Once growth was established and the medium became acid, modified Earle's solution containing 6 per cent lamb serum was used as the replacement medium.

When the cells in the dilution bottles reached

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* Obtained through the courtesy of Dr. Lennette of the California Public Health laboratory; originally obtained (Nov., 1954) from Microbiological Associates, Bethesda, Md.

† The photographs were taken with 16-mm. micro-Tessar lens through rounded test tubes, which accounts for some material not being in focus.

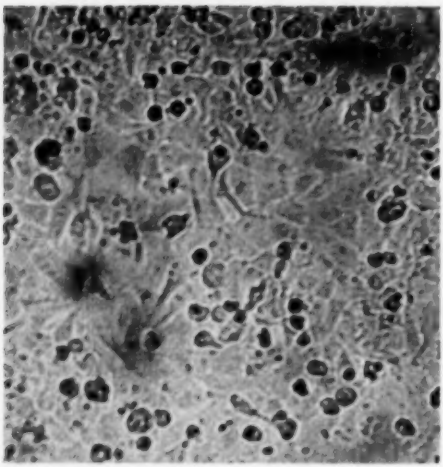

**Fig. 2—Confluent sheath of Hela cells not infected with NDV. Approximately x 133.**

maximum growth, they were harvested with versene, washed with a buffered salt solution free of calcium and magnesium ions and, finally, suspended in modified Earle's solution containing 10 per cent human serum. The cell suspension was then dispensed in 2.0-ml. amounts in 16- by 125-ml. Pyrex test tubes.

*Virus.*—The GB strain of NDV used in this study was obtained from Dr. G. Boney of Texas. The initial inoculum for the tissue cultures consisted of 0.25 ml. undiluted allantoic and amniotic fluids of embryonating chicken eggs having an e.l.d.$_{50}$ titer of $10^8$.

The TC strain of NDV consisted of the supernatant fluid of the seventy-seventh serial passage of the virus through Simms-Sanders medium containing chopped chicken embryo. This strain was demonstrated to be lethal for chicken embryos, but apathogenic for chickens.[2]

## Results and Discussion

In the initial serial passages, neither strain GB nor strain TC of NDV produced cytopathogenic changes which were visible macroscopically in the Hela or bovine kidney cell cultures. Under low-power examination (100 x), changes characterized by destruction of the cellular outgrowths, particularly along the margins of the cell sheath, were observed in the first Hela cell culture passage by the forty-eighth hour. No histological identification of cell types was attempted; however, the cytoplasm was granular and the cells were becoming rounded or swollen, seeming, in some instances, to have exploded and thus presenting bizarre shapes. There is a contrast between the appearance of uninfected and infected cells (fig. 1 and 2). Although passages were not made at regular intervals but only when the cytopathogenic activity

**TABLE 1—Summary of Serial Passages of Strains GB and TC of Newcastle Disease Virus Through Monolayer Cultures of Hela and Bovine Kidney Cells**

| Virus strain | Cell medium | No. passages | Infectivity of tissue culture: Passage No. | Infectivity of tissue culture: Chicken embryo * | Infectivity of tissue culture: Titer † | Remarks |
|---|---|---|---|---|---|---|
| GB | Hela | 20 | 1 | + | .... | |
| | | | 4 | + | .... | |
| | | | 8 | .... | $10^2$ | Marked cytopathogenicity |
| | | | 9 | .... | $10^4$ | observed by 14th passage. |
| | | | 12 | .... | $10^4$ | |
| | | | 14 | .... | $10^6$ | |
| | | | 18 | + | .... | |
| | Bovine kidney | 9 | 2 | .... | $10^6$ | |
| | | | 4 | .... | $10^2$ | Cytopathogenicity |
| | | | 6 | + | .... | observed on microscopic |
| | | | 7 | .... | $10^4$ | examination only. |
| | | | 9 | .... | $10^4$ | |
| TC | Hela | 20 | 5 | .... | $10^3$ | |
| | | | 8 | .... | $10^4$ | Marked cytopathogenicity |
| | | | 12 | .... | $10^4$ | by 17th passage |
| | | | 17 | .... | $10^6$ | |
| | | | 20 | .... | $10^6$ | |
| | Bovine kidney | 10 | 4 | .... | $10^2$ | Cytopathogenicity |
| | | | 7 | .... | $10^3$ | observed on microscopic |
| | | | 9 | .... | $10^4$ | examination only. |
| | | | 10 | .... | $10^3$ | |

* Supernatant fluid inoculated into each of 3 embryonating chicken eggs; diagnosis based on death, typical lesions, and hemagglutination of a chicken erythrocyte suspension by the allantoic-amniotic fluids of the embryos.

† Titration of supernatant fluid in embryonating chicken eggs conducted on cultures stored at −6 C.

was observed microscopically, the incubation periods of the inoculated cultures varied from two to ten days. When cytopathogenicity was not observed within 48 hours, the pH of the liquid medium was restored by a change to fresh medium, and the observations were continued. Once the virus seemed established, cytopathogenicity on Hela cells was easily discernible by the naked eye by the forty-eighth hour. Cell destruction that could be observed macroscopically occurred with strain GB by the fourteenth serial passage, and with strain TC by the seventeenth serial passage. This reaction was evident in each passage thereafter. The cytopathogenicity of each strain in the eighteenth serial passage is shown (fig. 3 and 4).

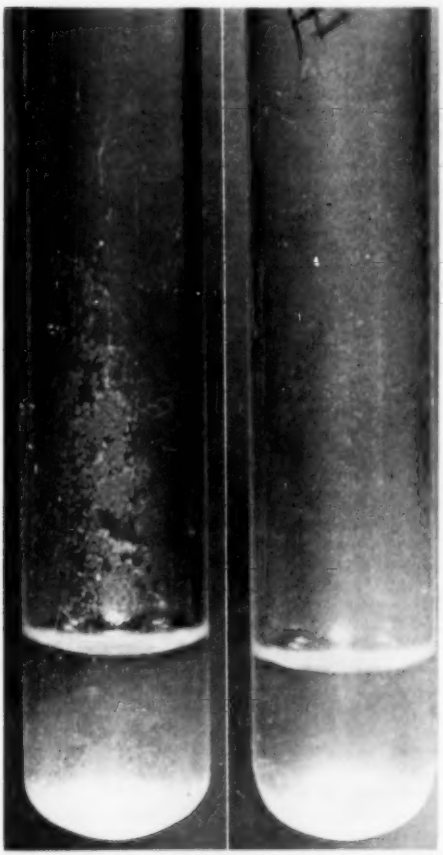

Fig. 3—Cytopathogenic effect of strain GB of NDV on Hela cells inoculated 48 hours previously with the eighteenth serial passage. Note the marked destruction and fragmentation of the sheath of the inoculated tube (left) when compared with the uninoculated control tissue culture (right).

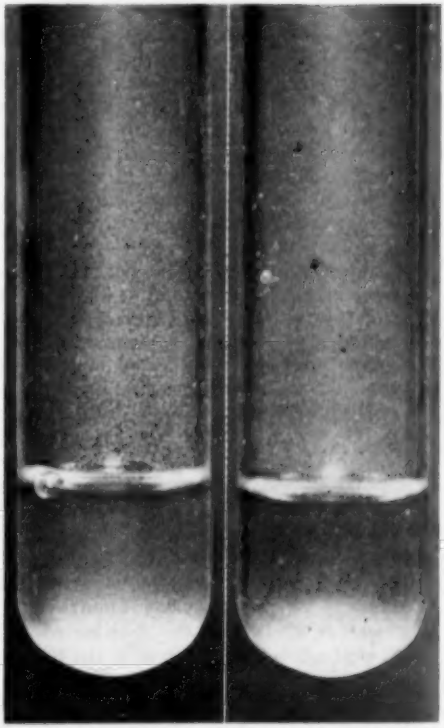

Fig. 4—Cytopathogenic effect of strain TC of NDV on Hela cell culture inoculated 48 hours previously with the eighteenth serial passage. Note the activity of the virus on the cell sheath (left) when compared with the uninoculated control tissue culture (right) and the relatively mild cytopathogenicity when compared with figure 3.

Marked differences in degree of cell destruction were produced by each strain. Strain GB appeared to destroy larger areas of tissues in the sheath. The cytopathogenicity of the TC strain, however, was considerably less marked. When cultures of the more virulent strain were allowed to incubate for 96 hours, all of the cells in the tube were destroyed; the TC strain destroyed only about half the cells.

Both strains of the virus were also cultivated in bovine kidney tissue cultures. Microscopically, the cytopathogenic effects, unlike those observed on the Hela cell cultures, were readily detected in the initial passages. The degree of cell destruction by both strains of the virus in the ten serial passages was not as obvious as that observed in Hela cells. In addition, the

lower degree of invasiveness and cytopathogenicity of the TC strain, as compared with the GB strain, was also observed in the bovine kidney cell cultures. The milder activity of these two strains of the virus upon the kidney cells was due to milder degenerative changes in cell morphology, the degree of inhibition of cell migration, and changes in tissue metabolism as indicated by decreased acid production.

As shown (table 1), both tissues supported growth and propagation of the two strains of NDV; however, the destruction of the cell sheath, which could be detected by the naked eye, did not appear in the Hela cell cultures until the fourteenth and seventeenth passages of strains GB and TC, respectively. The cause for this delay has not been determined; it may be due to a period of adaptation of the virus to the new environment which gradually resulted in higher concentrations of the virus on serial passage. Once this activity on the cells was established, no difficulty was encountered in reproducing the effect in test tubes or in preparing larger quantities of the virus in Roux's flasks. Similar results could perhaps be obtained with the bovine kidney cells if the passages were continued. The advantages of using a known line of cells, such as the Hela cell, in place of cell cultures originating from unknown individual animals from varied sources were considered great enough to discontinue the latter method.

## SUMMARY

Two strains of Newcastle disease virus, the highly pathogenic GB and an avirulent tissue culture-attenuated NDV, were propagated in monolayer cultures of Hela and bovine kidney cells. The degrees of cytopathogenicity, as demonstrated macroscopically by the eighteenth serial passage in both tissues, were correlated with their relative virulence for chickens.

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# Pathological Changes Associated with *Dictyocaulus Viviparus* (Bloch) Infections in Calves

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LUNGWORM DISEASE, caused by *Dictyocaulus viviparus*, is a rather common parasitic infection which causes considerable mortality in young calves. Earlier reports on this disease have been primarily concerned with the pathological changes of initial infections.

This paper reports the results of an investigation in which three forms of lungworm infection in calves were studied. These forms include: (1) initial infections, (2) recovered cases, and (3) recovered cases subsequently challenged with viable lungworm larvae.

## REVIEW OF LITERATURE

The clinical signs of *D. viviparus* infections in cattle include respiratory distress, nasal and oral discharge, lacrimation, diarrhea, anorexia, rough coat, emaciation, and debility.[2, 5, 7, 9, 10, 12, 17]

The principal postmortem lesions[2, 5, 7, 9, 12] in calves are found in the lungs and adjacent lymph nodes. Pneumonia is evident in longstanding cases and varies considerably in extent; whereas, in acute infections (7 days), it is absent. Enlargement of the lungs due to interstitial and subpleural emphysema and edema has been described. The trachea, bronchi, and bronchioles may contain a frothy fluid mixed with lungworms. The bronchial and mediastinal lymph nodes are usually edematous or hyperemic.

Histological examination of lungs demonstrating consolidation reveals that the alveoli contain a cellular exudate consisting of neutrophils, phagocytes, lymphocytes, foreign body-type giant cells, eosinophils and, occasionally, lungworm larvae. The bronchi and bronchioles show epithelial thickening with exudate consisting of desquamated epithelial cells, degenerated cells, neutrophils, lymphocytes, phagocytes, and lungworms. Lymphoid hyperplasia adjacent to bronchi and bronchioles is frequently reported. Interstitial emphysema is is a common finding. On the basis of histopathological findings, lungworm disease is designated as a verminous bronchopneumonia.[1, 8-9, 11, 13, 15]

Several authors have shown that recovery from lungworm disease confers almost complete resistance to reinfection.[2-6, 8, 11, 12] Patent reinfections were rarely observed. In those instances which were seen, only a small number of immature worms were found in the lungs, and mild respiratory changes were observed.[5]

## MATERIALS AND METHODS

Of the 10 calves utilized in these experiments, 9 were purchased at 3 to 8 days of age and raised, free of lungworms, in individual pens. Calves 1, 2, 4, 5, 7, and 10 were used to investigate the effect of initial infections and calves 21 and 26 were employed in the challenge study. Calves 6 and 4L were used in the study of recovered animals. The latter calf (4L) was a naturally infected field case. Six control calves were maintained for weight comparison studies.

First-stage larvae, obtained from feces with the Baermann apparatus, were cultured to the third stage in petri dishes containing tap water and granular animal charcoal. These third-stage, infective larvae were washed from the charcoal immediately prior to administration. Larval counts were made from aliquot samples and motile or coiled larvae having a transparent, glistening appearance were classified as living. Opaque, slightly curved larvae containing many undigested food granules were considered dead. Differentiation of live and dead larvae did not depend solely upon motility.

All experimental infections were produced by the oral administration of infective larvae, 6 to 36 days old. Initial doses were given daily over a period of four to five days. Challenge doses were administered as a single drench or as two drenches, eight days apart.

Clinical examinations were conducted daily in the case of initial infections. All the calves were examined three to seven times weekly, with the exception of the one with the field infection, which was examined as frequently as deemed necessary. Fecal specimens were taken directly from the rectum and a 20-Gm. sample examined for larvae with a Baermann apparatus. At necropsy, the respiratory tract was opened with scissors from the larynx to the small bronchioles. Macroscopic worms were removed, placed in physiological saline solution, and counted. To find the number of microscopic worms present, the respiratory passageway was rinsed over a 100-mesh screen. The lung tissue was cut in small cubes of 1 cubic inch or less, submerged in large containers of physiological saline solution, and incubated at 37 C. overnight. The lung tissue was then rinsed and the saline suspension washed over a 100-mesh screen. The material on the screens was resuspended in a known volume of saline solution (usually 1 liter) and the number of microscopic worms estimated from the average

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number of worms found in three 20-ml. aliquots of this suspension. The first stage larvae readily passed through the 100-mesh screen and, therefore, did not enter into the count.

Sections for microscopic study were routinely collected from the bronchial and mediastinal lymph nodes and lungs of all calves at necropsy. Occasionally, sections of liver, spleen, kidney, heart, and ruminal lymph nodes were also saved for histological study. All tissues were fixed in 10 per cent neutral formalin, cut at 6 $\mu$, and stained with hematoxylin-eosin. Van Gieson's stain was employed where indicated.

## RESULTS

*Initial Infections.*—Clinical Changes.—On postinfection days 18 and 28, 2 animals infected with 70,000 larvae died of acute lungworm disease. A third animal, after receiving the same number of larvae, became moribund on day 29 and was killed (table 1). The average prepatent period in 3 animals which were given 5,000 larvae was 25.3 days; the maximum number of larvae recovered from the feces of these calves varied from 8 to 62 per Gm. of feces.

The signs of lungworm disease in all calves were primarily respiratory in nature, with marked loss of condition and weight in animals with acute infection. The average weight loss in acutely sick animals during the second week was 47 lb. Calves infected with 5,000 larvae gained steadily in weight and showed only mild retardation in normal expected weight gains during the first three weeks of infection, when compared with control animals.

A pronounced increase in respiratory rate was first observed between days 8 and 9 in the animals infected with 70,000 larvae and between days 13 and 15 in those infected with 5,000 larvae. Coughing of variable severity was first noticed nine to 12 days after infection and was intermittent during the course of infection. Acute cases were characterized by a wheezy, moist cough, bubbling râles, expiratory grunt, nasal discharge, and salivation. In nonfatal infections, the cough was dry and without dyspnea.

In acute infections, temperatures either remained normal or became elevated at the first sign of respiratory symptoms and remained so until shortly before death. Diarrhea and anorexia were noticed on postinfection days 8 and 9. Constipation associated with dehydration followed, and the animals remained anoretic until death.

Gross Lesions.—Enlargement of the lungs was a constant finding in all animals. Pneumonic areas, which were bilateral and involved the cardiac, apical, and diaphragmatic lobes, were more extensive in calves killed on postinfection day 43 than in those which died on days 28 and 29. Gross evidence of pneumonia was lacking in the animal which died on day 18. However, hemorrhagic bronchitis, blood clots in the bronchioles, and immature lungworms concealed in froth in the trachea and bronchi were found. Small spaces were seen in the mediastinal lymph nodes in animals with 18- to 20-day infections. Bronchial and mediastinal lymph nodes were enlarged in all cases.

Worm counts ranging from 10,625 to 19,255 were obtained at necropsy from calves which had been given 70,000 larvae (table 1). The 3 calves which had been infected with 5,000 larvae were found to harbor 17, 120, and 308 worms.

Microscopic Lesions.—Pulmonic lesions were similar in calves 1, 2, and 4. The pleura and interlobular septums were remarkably thickened due to edema, fibrinous exudate, infiltration with eosinophils, and spectacular dilatation of the lymph vessels. The enlarged lymph channels contained lymph, lymphocytes, eosinophils, some fibrin and, occasionally, giant cells and mononuclear cell macrophages. Rarely, the lymphatics contained an organized thrombus (fig. 1). Pneumonic consolidation usually involved entire lobules. The alveolar walls appeared thickened and the

**TABLE 1—Data on Initial Dictyocaulus Viviparus Infections in Calves**

| Calf No. | Age at time of infection (days) | No. larvae administered | Prepatent period (days) | Peak larvae per Gm. feces | Duration of infection (days) | Total worms recovered at necropsy |
|---|---|---|---|---|---|---|
| 1 | 133 | 5,000† | 28 | 25 | 43§ | 120 |
| 2 | 133 | 5,000† | 25 | 8 | 43§ | 17 |
| 4 | 133 | 5,000† | 23 | 62 | 43§ | 308 |
| 5 | 210 | 70,000‡ | ‖ | .... | 18‖ | 10,625 |
| 7 | 203 | 70,000‡ | ‖ | .... | 28‖ | 19,255 |
| 10 | 203 | 70,000‡ | * | .... | 29* | 16,032 |

* Killed in extremis; † over a period of 5 days; ‡ over a period of 4 days; § killed; ‖ died.

alveoli were filled with exudate consisting primarily of alveolar-lining cells (macrophages) with and without phagocytized cells, eosinophils, and giant cells which were occasionally bizarre-shaped. Lymphocytes, neutrophils, plasma cells, erythrocytes, and some edematous fluid were, infrequently, constituents of the exudate. A few alveolar-lining cells were undergoing degeneration as indicated by pyknosis of the nuclei and intracytoplasmic vacuolization. Plugs consisting of necrotic debris and eosinophils often completely occluded the bronchi and large bronchioles (fig. 2). Peribronchial, peribronchiolar, and perivascular cuffing with lymphocytes often occurred. In such instances, the muscular tunic and tunica propria of bronchioles were also infiltrated with lymphocytes, along with eosinophilic and lymphocytic infiltration of the epithelium. Desquamation or hyperplasia of the bronchiolar epithelium also occurred. Small bronchioles were generally collapsed or ruptured, and usually contained a few eosinophils or a small amount of eosinophilic debris. Larvae were seen in the bronchi, bronchioles, and alveoli (fig. 3). An occasional larva in the alveoli was undergoing phagocytosis (fig. 4).

The mediastinal and bronchial lymph nodes of calves 1, 2, and 4 displayed lymphoid hyperplasia and eosinophilic infiltration of the subcapsular region and trabeculae. The same lymph nodes of calves 5, 7, and 10 contained distended lymph sinuses in the subcapsular region and cortical zone (fig. 5). Small hemorrhages were scattered throughout the nodes.

The pneumonic lesions in animals 7 and 10 were similar. Pneumonic lobules contained varying degrees of functional tissue, and the entire lobules were rarely affected. Consolidation of the alveoli was characterized by a cellular exudate composed primarily of alveolar-lining cells, giant cells, lymphocytes, and a few neutrophils. Occasionally, these cells were found in a matrix of fibrin and edematous fluid, and, infrequently, two giant cells filled an entire alveolus. Bronchioles in such consolidated areas contained varying amounts of cellular exudate consisting of desquamated epithelial cells, unidentifiable degenerated cells, and a few erythrocytes, but they were rarely occluded. Frequently, such bronchioles exhibited epithelial hyperplasia (fig. 6). Larvae were observed in bronchioles and alveoli. In more mildly involved lobules, there was alveolar emphysema and peribronchiolar pneumonic consolidation.

Microscopic lesions in the bronchial and mediastinal lymph nodes of calf 5 were similar to those described in animals 7 and 10. In sections of lung of calf 5, the alveolar walls were ruptured or the interalveolar septums were thin. Many small bronchioles were ruptured, collapsed, or distorted, while larger bronchioles occa-

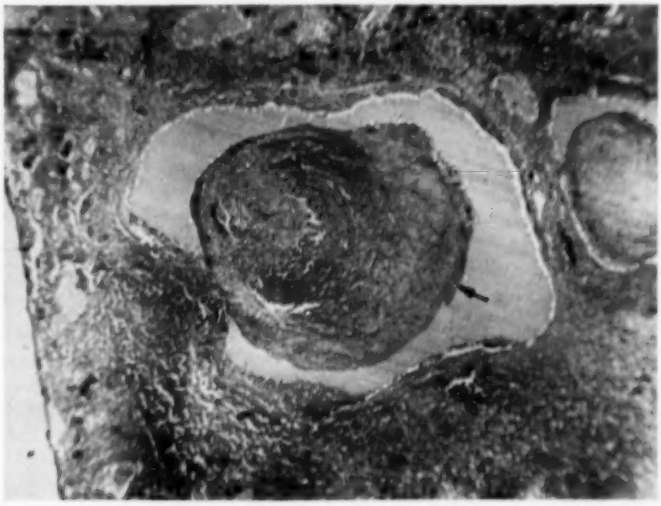

**Fig 1 — Thrombus (arrow) in a dilated lymph-filled lymphatic in the interlobular septum of the lung of calf 4. The other large spaces are also dilated lymphatics. H & E stain; x 16.**

sionally showed epithelial desquamation and usually contained necrotic debris without being occluded. There was peribronchial and peribronchiolar cuffing by eosinophils. Large, gas-filled lymphatics occurred in the pleura and interlobular septums. Immature lungworms were seen in the large branchioles and in a blood clot removed from a bronchiole at necropsy.

*Infections in Recovered Animals.*—Clinical Changes.—This experiment involved a study of calf 6, which was infected with 50,000 larvae over a period of four days, and also calf 4L—a field case. Typical clinical signs of lungworm disease were seen in both animals but the infection was more severe in calf 6. The peak larval output was 508 per gram of feces in calf 6 and 199 per gram in 4L. When killed, approximately four months after recovery, their status of recovery being determined by inability to obtain larvae from the feces, there was no evidence of respiratory difficulties in either calf.

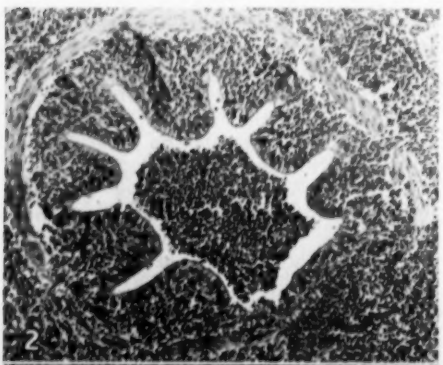


Fig. 2—Bronchiole showing partial occlusion by necrotic debris and eosinophils (calf 1). H & E stain; x 66.

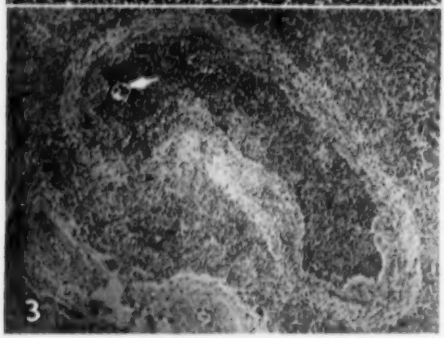


Fig. 3—Bronchiole (calf 4) showing occlusion by necrotic debris. Note larva (arrow) entangled in the necrotic material. H & E stain; x 20.

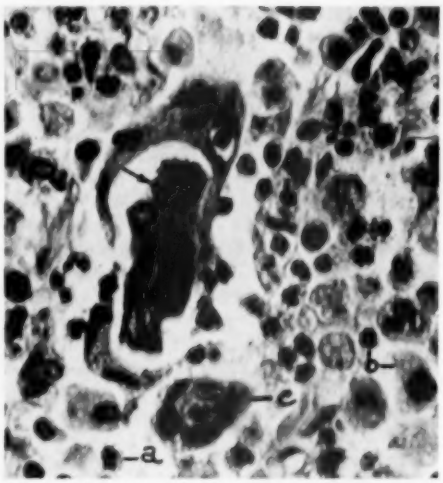


Fig. 4—Lungworm larva (arrow) being phagocytized by a giant cell (calf 4). Some alveolar exudate is identified: an eosinophil (a), an alveolar-lining cell (b), and a giant cell (c). H & E stain; x 65.

Gross Lesions.—The lungs of calf 6 appeared normal except for a well encapsulated abscess, 18 mm. in diameter, in the left apical lobe. Lungworms were not recovered from the lungs. A dorsal mediastinal lymph node contained a well-demarcated, subcapsular lesion 2 mm. in diameter which grossly resembled a blister except that it did not contain fluid. Many similar minute lesions were distributed throughout the node. The bronchial nodes were hemorrhagic. There was a complete absence of gross lesions in the lungs and lymph nodes of calf 4L, although 5 immature lungworms were recovered from the lungs.

Microscopic Lesions.—In sections made from the apical lobe of the left lung of calf 6, but taken some distance from the abscess, small bronchioles showed evidence of chronic inflammation and usually contained fine basophilic, granular debris. These bronchioles, showing desquamation or degeneration of the epithelium, were often ruptured and there was muscular hypertrophy (fig. 7). Large bronchioles revealed lymphocytic infiltration of the tunica propria and peribronchiolar cuffing with lymphocytes. Dead ova, approximately 70 $\mu$ in diameter, and dead larvae were seen in the bronchi, bronchioles, and

**TABLE 2—Data on 2 Animals Challenged with Lungworm Larvae Following Recovery from Initial Infection**

| | Initial infection | | | | | Challenge infection | | |
|---|---|---|---|---|---|---|---|---|
| Calf No. | Age at time of infection (days) | No. larvae administered | Prepatent period (days) | Patent period (days) | Peak larvae per Gm. feces | Days between challenge and necropsy | No. of larvae administered | Total worms recovered at necropsy |
| 21 | 133 | 12,000 | 28 | 31 | 1 | 68 | 33,000 | 0 |
| 26 | 156 | 61,000 | 25 | 38 | 30 | 36 | 82,000 | 0 |

alveoli. The ova were stained purple with hematoxylin and eosin and, infrequently, contained a visible larva. Usually, the ova were unattended by a cellular reaction except, rarely, when observed in the mucous glands of the bronchi surrounded by lymphocytes. The cardiac and diaphragmatic lobes showed the same lesions of the bronchioles as were seen in the apical lobe. In all lobes, the alveolar walls were thickened by an infiltration by lymphocytes and fibroblasts. Little exudate was seen in the alveoli. The mediastinal lymph nodes contained dilated lymph sinuses, while the bronchial nodes displayed scattered, large hemorrhages.

The lungs of calf 4L demonstrated almost complete microscopic resolution of pneumonia. Some small bronchioles were ruptured and the epithelium was desquamated. A few larger bronchioles were mildly cuffed with lymphocytes, and a small amount of necrotic debris was infrequently found in the lumina. Scant cellular exudate, primarily alveolar-lining cells, was found in the alveoli adjacent to affected bronchioles. The pleura was slightly thickened.

*Infections in Recovered Animals Following Challenge.*—Calves 21 and 26, after being initially infected with 12,000 and 61,000 lungworm larvae, respectively, developed typical signs of lungworm disease.

At the time of recovery, they were challenged with viable larvae (table 2). Calf 21 was given 33,000 larvae in a single dose; calf 26 was given 68,000 larvae followed, eight days later, by 14,000 more. No clinical evidence of lungworm disease resulted.

Gross Lesions.—The lungs of calves 21 and 26 lacked normal elasticity and contained small, white nodules. The lymph nodes of calf 21 were normal, whereas in calf 26 the mediastinal nodes were enlarged and the ruminal nodes contained cysts filled with watery fluid. Lungworms were not recovered from the lymph nodes or lung tissue of either calf.

Microscopic Lesions.—The interlobular septums of the lungs of calf 21 were slightly thickened due to productive tissue changes. The alveolar walls were infiltrated by fibro-

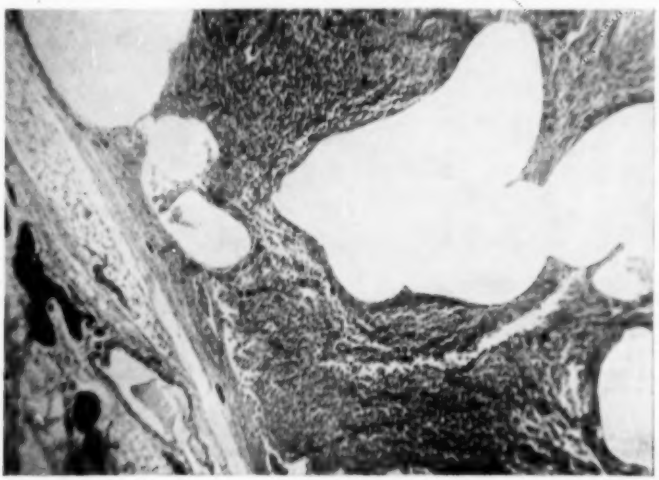

**Fig. 5—Mediastinal lymph nodes (calf 7) showing distended lymph sinuses. H & E stain; x 16.**

blasts and lymphocytes. Isolated nodules, composed of lymphocytes, rarely with eosinophils at the center, occurred throughout the lung (fig. 8). Large bronchioles and bronchi usually contained basophilic debris and often displayed epithelial desquamation or hyperplasia. Lymphocytic infiltration, uncommonly limited to the lamina propria of bronchioles, extended into the peribronchiolar area (fig. 9). Small bronchioles, having desquamated or degenerated epithelium, were collapsed or ruptured and there was muscular hypertrophy of their walls.

Pathological changes in the lungs of calf 26 were similar to those already described for calf 21, except that lymphoid hyperplasia about the bronchioles was frequently so extensive that all that remained of the respiratory structure was a degenerated epithelial lining. The subcapsular area of the mediastinal node was infiltrated with eosinophils. In the germinal center of the lymph nodules, basophilic-stained bodies were seen, which, when compared to immature lungworms, appeared to be dead

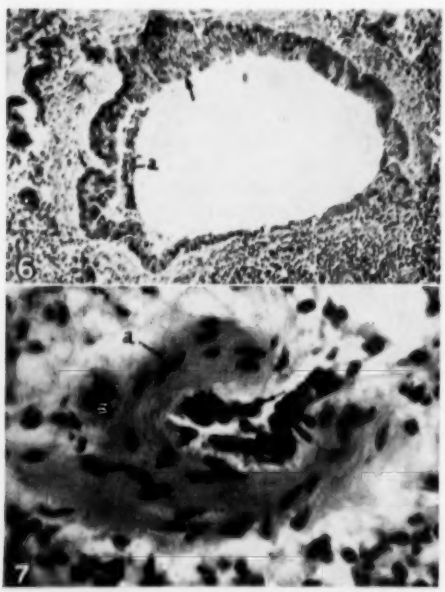

Fig. 6—Bronchiole with epithelial hyperplasia (arrow) from calf 7. A small amount of debris (a) is seen in the lumen of the bronchiole. H & E stain; x 55.

Fig. 7—A bronchiole with muscular hypertrophy (a) and degenerated epithelium (arrow) from calf 6. H & E stain; x 265.

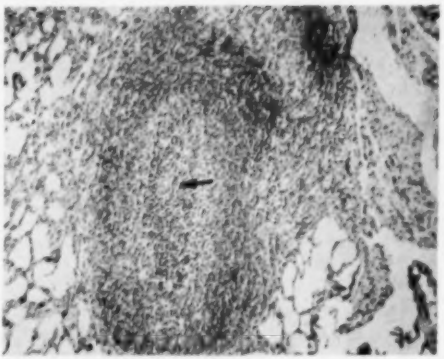

Fig. 8—Lung from calf 21, containing nodule composed of lymphocytes with eosinophils (arrow) at center. H & E stain; x 66.

larvae (fig. 10). Occasionally, the larvae were within multinucleated giant cells. Hemosiderin-laden macrophages were frequently found in the lymph follicles of the mediastinal node. The ruminal node was devoid of lymphoid tissue, and was composed of strands of fibrous connective tissue separated by large cystic areas.

## DISCUSSION

Respiratory symptoms of the acute, fatal lungworm infections reported in this paper can be attributed to the grossly evident froth mixed with immature lungworms in the entire respiratory passageway, and to the microscopically evident alveolar and interstitial emphysema.

This study indicates that the absence of pneumonia and of adult lungworms at necropsy does not necessarily eliminate the possibility of lungworm disease as the cause of death in young calves. Under field conditions, enlargement of the lungs and the presence of froth in the respiratory passageways should suggest the possibility of death due to the acute, fatal form of lungworm disease. Immature lungworms mixed with froth are difficult to see in the respiratory tract but can be readily detected by collecting the washings from the larger air passages in a glass container and holding the suspension in front of a strong light.

Histological sections demonstrated that the extent and type of pathological changes in the lung varied with the duration and severity of infections. The acute nature of the disease, in an infection which terminated fatally in 18 days, accounted for the

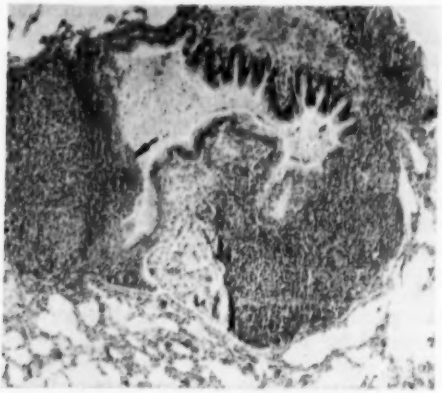

Fig. 9—Section of lung (calf 21) showing peribronchiolar lymphoid hyperplasia. Note epithelial desquamation (arrow). H & E stain; x 40.

absence of gross evidence of pneumonia. The immediate cause of death in this animal (5) was anoxia. Nonfatal cases of long duration were characterized by extensive lung consolidation which was accompanied by pleural and interlobular edema.

Acquired resistance to lungworm disease following recovery [3, 4, 6, 8, 11, 13, 14] has been reported. This paper substantiates that finding, since clinical evidence of disease was not observed in recovered animals following challenge, nor were living lungworms found in the lungs at necropsy.

The phenomenon of inhibited development of larvae has been described.[4-6, 11, 16] The results reported herein substantiate this work, since 1 recovered animal was found to harbor 5 immature worms in the lungs at necropsy and dead larvae and ova were seen in histological sections of lungs of a second recovered animal.

Microscopic observations permitted a study of the pathogenesis of the pneumonia of lungworm disease in calves. In acute cases, bronchitis and bronchiolitis, in addition to the mechanical obstruction incurred by large numbers of immature worms, caused the air passageways to become partially occluded and stimulated the development of extensive alveolar and interstitial emphysema, resulting in death due to anoxia.

The bronchitis and bronchiolitis of calves with nonfatal cases were not complicated by the extreme mechanical obstruction of the respiratory passages with numerous larvae and froth as occurred in animals with the acute infection. Cuffing of the bronchi and bronchioles by lymphocytes followed inflammation of these parts. The inflammatory process continued into the adjacent alveoli and the pneumonic condi-

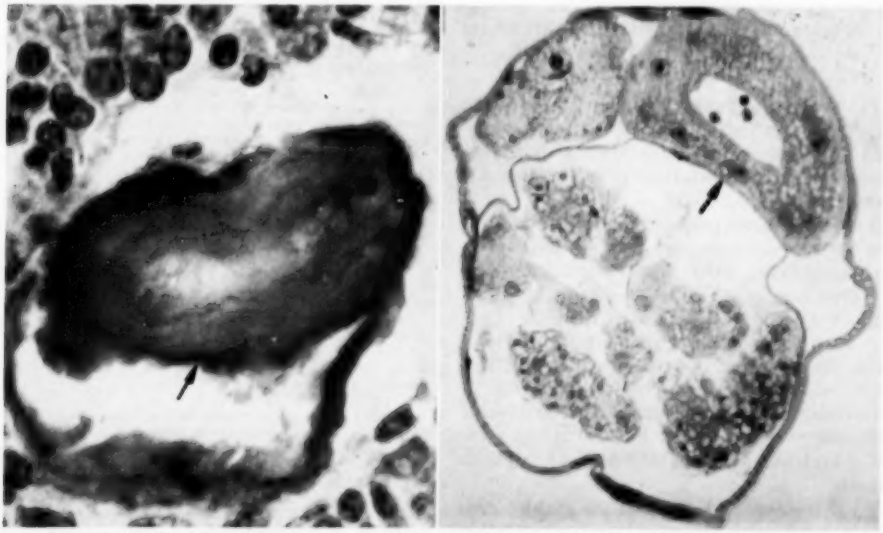

Fig. 10 (Left)—Dead larva in mediastinal lymph gland (calf 26). H & E stain; x 560. (Right)—Cross section of immature lungworm obtained from an experimental case. Note gut of parasites (arrow) in each picture. H & E stain; x 475.

tion spread until entire or almost entire lobules were involved.

Evidence of beginning resolution of pneumonia appeared in lobules which were completely consolidated. Ingestion of larvae by giant cells, the appearance of alveolar-lining cells with cytoplasmic vacuolization, and pyknosis of nuclei were the first signs of resolution. Apparently, resolution was nearly complete four months following the last detection of larvae in the feces in calf 4L, since at necropsy only slight bronchitis, bronchiolitis, and scant cellular exudate in the alveoli were seen. Lack of complete resolution was not surprising since 5 immature lungworms were found in the lungs at necropsy. Resolution was not complete in calf 6. There was a macroscopic abscess in the apical lobe, plus productive tissue changes in the alveolar walls and muscular hypertrophy of the bronchiolar wall. This animal experienced a more severe initial infection than calf 4L as indicated by clinical signs and fecal larvae. Consequently, lung damage was probably greater in calf 6, resulting in permanent injury.

The histological study of lungs of calves which were challenged following recovery showed chronic inflammatory changes in the bronchioles and alveolar septal walls with peribronchiolar lymphoid hyperplasia. The appearance of dead larvae in the mediastinal lymph node indicates that larvae can enter and be phagocytized in this node.

## SUMMARY

1) The acute form of lungworm disease resulted from infection with massive numbers of larvae (e.g., 70,000) while nonfatal cases resulted from infection with small numbers (e.g., 5,000).

2) Extensive pneumonia involving entire lobules occurred in animals with nonfatal infections. Alveolar exudate consisted of: alveolar-lining cells, eosinophils, giant cells, lymphocytes, and neutrophils. Larvae were seen in the alveoli. Cellular exudate, necrotic debris, and larvae frequently occluded the large bronchioles and bronchi. Thickening of the pleura and interlobular septums was due to edema, fibrinous exudate, and marked dilatation of the lymph vessels.

3) Alveolar and interstitial emphysema was a constant histological finding in acute infections. A calf with an infection of short duration (e.g., 18 days), did not display microscopic evidence of pneumonia, but small bronchioles were ruptured, collapsed, or distorted. Large bronchioles contained necrotic debris, larvae, and desquamated epithelial cells. Death resulted from anoxia. Calves with acute cases of longer duration (e.g., 28 to 29 days), displayed pneumonia which was primarily peribronchiolar in location and rarely involved entire lobules. The mediastinal lymph glands contained dilated lymph sinuses.

4) Histological sections indicated that the pneumonia of lungworm disease was initiated by a bronchitis and bronchiolitis which continued into the adjacent alveoli.

5) The resolution of pneumonia of lungworm disease was practically complete in 1 calf killed for necropsy approximately four months after recovery. Scant alveolar exudate and cuffing of bronchioles by lymphocytes were observed. Productive tissue changes and dead larvae and ova were seen in sections of lung of a second recovered calf.

6) Nodules of lymphoid tissue, peribronchiolar lymphoid hyperplasia, bronchiolar muscular hypertrophy, and productive tissue changes in the alveolar septal walls and interlobular septums were observed in the lungs of 2 calves killed following recovery and challenge with lungworm larvae. In 1 of these calves, dead larvae were found in the germinal centers of lymph nodules of the mediastinal lymph node.

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# Effectiveness of Various Measures in Bloat Therapy and Prophylaxis, and the Resulting Influence on Rumen Fluid Characteristics

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BLOAT PREVENTION and treatment have been problems for centuries. Many of the managemental measures which are practiced today were mentioned in early eighteenth century literature.[2] In spite of extensive modern knowledge of the bloat syndrome, over 50 treatment and preventive measures have been advocated recently in scientific literature.

Recently suggested treatments can be categorized as follows: defoaming agents, hydrogen-ion alterants, a saponin-precipitant, antibiotics, and neuromuscular agents. Agents such as methyl silicones, fatty acids, coal tar preparations, oils, saliva, detergents, and cream have been suggested as being effective in reducing bloat by releasing the foam-trapped gas. Experiments comparing the defoaming effectiveness of a large number of agents *in vitro* have been conducted by Brunner,[5] and by Dougherty and Meredith.[6] It is now known that such agents also decrease rumen fluid surface tension both *in vivo* and *in vitro*.[3] The relationship between decreased surface tension and foam dispersion, in regard to bloat therapy, is not well understood. Nichols[12] has proposed that detergents increase bubble size in frothy bloat, thus allowing the larger bubbles to rise and become more available for gas liberation. It has been suggested[17] that changes in ruminal pH affect eructation efficiency; therefore, hydrogen-ion alterants may have therapeutic value in bloat. Cholesterol, a saponin precipitant, largely counteracted the chick growth-depressing action of alfalfa meal.[13] It also has been investigated as a bloat prophylactic.[9] Although there are conflicting scientific reports, antibiotics have been reported as having therapeutic[6] and prophylactic[1] value against certain types of bloat. The mechanism of action of antibiotics on bloat is unknown; however, it probably is not associated with surface tension.[16] A "solution" of calcium, magnesium, and phosphorus has been reported of value in relieving bloat, possibly by correcting an eructation malfunction.[14]

Many managemental recommendations for preventing bloat have been suggested in recent years. However, the majority stem from the "physical stimulus" theory of eructation.[7] Although some research fails to support this theory, these recommendations should not be ignored until better preventive measures or a more ideal medicament can be demonstrated.

The objectives of the present research were to evaluate the effect of a number of agents on the bloat syndrome and to study the interrelationships between rumen fluid characteristics and bloat pathogenesis.

## EXPERIMENTAL PROCEDURE

Two experiments were conducted: (1) an alfalfa grazing trial, wherein an alkyl aryl sodium sulfonate-type detergent (Ultrawet "K"*) and a pH alterant (trisodium phosphate) were evaluated prophylactically, and (2) a study of the bloat-preventive effectiveness of the detergent and of cholesterol when added to bloat-provocative forage extracts.

In both experiments, daily multiple observations, or tympanometer† measurements, or both were employed as criteria of the effectiveness of the therapy or prophylaxis. Bloat severity was rated from 0 (no bloat) to 5 (severe bloat). Bloat incidence was recorded as the percentage of bloat cases in relation to the total number of observations made.

Evaluations were made on rumen fluids and, in some cases, on plant juices or rations. Each surface tension value, determined with a Cenco-duNouy interfacial tensiometer, was the mean of at least five consecutive readings. Hydrogen-ion concentration was measured with a model H2 Beckman pH meter. Viscosity was measured by comparing the rapidity with which 15 ml. of sample and 15 ml. of distilled water, respectively, flowed from a 25-ml. serological pipette stationed vertically and filled to the top (0 mark). The sample-to-water time ratio, with water arbitrarily assigned a value of 1, indicated the relative sample viscosity. Specific gravity was measured by comparing the weight of a 25-ml. sample with 25 ml. of distilled water. Ingesta volume increase (IVI) was measured in a 50-ml. graduated cylinder containing 20 ml. of sample incubated at 37 C. until peak volume occurred. Foam half life was determined by measuring the time required for dispersion of one half of the foam in an artificial foam column produced by agitating a 30-ml. sample for two minutes in a Waring Blendor. The degree of foaminess (foam index) of the fresh samples was

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* Supplied by the Atlantic Refining Co., Philadelphia.

† An instrument for measuring intraruminal pressure.[11]

expressed as 0, no foam; 1, slightly foamy; 2, moderately foamy; or 3, very foamy.

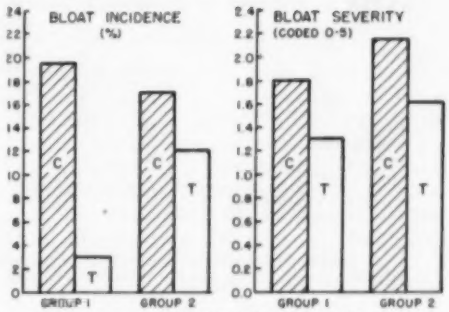
EXPERIMENT 1—EFFECT OF DETERGENT AND OF TRISODIUM PHOSPHATE ON BLOAT IN CATTLE GRAZING ALFALFA

*Procedure.*—Two groups, with 11 beef cattle (yearling heifers and cows) in each, were maintained on alfalfa pasture continuously during the summer of 1955. The field was divided into seven plots which were grazed in rotation to supply succulent forage continuously. Water and salt were available ad libitum. One group served as a control while the other received in succession, per 1,000 lb. of body weight, 30 Gm. of trisodium phosphate, 20 Gm. of detergent, and 30 Gm. of detergent. Subsequently, the groups were reversed and the former control group received the detergent at the 30-Gm. level. A control (adjustment) period of a minimum of three days preceded each treatment. All treatments were administered orally, by gelatin capsule. In addition to the bloat observations, grazing habits and weather conditions were recorded. Periodically, rumen fluid samples were collected from all animals, and surface tension, pH, foam half life, IVI, viscosity, specific gravity, and degree of foaminess were determined.

*Results and Discussion.*—One of the more difficult evaluations in grazing experiments of this nature is the quantitative estimation of bloat severity. Ordinarily, visual observation, palpation, and mechanical devices can be employed. However, in this trial, only the visual observations could be used because many of the experimental subjects were not tame, and it was desirable to minimize interference with grazing habits. To reduce variations in estimates, only two persons made the observations, and each followed the same evaluation guide.

The detergent had little effect at the lower dosage level; however, at the higher level a significant [4] ($P<0.01$) reduction in both bloat severity and bloat incidence occurred (fig. 1). Although there was some indication that trisodium phosphate reduced bloat slightly, results were much less distinct than with detergent, and additional data would be necessary to determine the extent of reduction. Nichols [12] has reported successful prophylactic use of detergents at a lower daily dosage level than that found to be necessary for effectiveness in this trial. Before adequate evaluations can be made on the use of the detergent as a prophylactic for pasture-type bloat, further consideration should be given to palatability, administration technique, dosage level, and cost.

A difference in various physical properties of rumen fluid, collected periodically from the grazing cattle, was observed between bloated and contemporary nonbloated animals, individual animals when bloated and when not bloated, and detergent-treated and nontreated animals. The IVI, foam durability (half life), and degree of foaminess (foam index) were markedly increased for ingesta from bloated animals and were oppositely influenced by prophylactic administration of the detergent (table 1). Similar, but less marked, changes were observed for surface tension and viscosity. Specific gravity and pH were lower during bloat and were increased by administration of the detergent. Trisodium phosphate apparently had little effect on rumen fluid properties.


BLOAT INCIDENCE
(%)
BLOAT SEVERITY
(CODED 0-5)
C
T
GROUP 1
GROUP 2
Bloat incidence = No. times bloat observed x 100 / total observations;
severity: 0 = no bloat, 5 = severe bloat; C = control; T = treated.

**Graph 1—Prophylactic influence of a detergent (30 Gm./1,000 lb. body weight daily) on alfalfa pasture bloat. Each group was composed of 11 animals.**

The effect of bloat on rumen fluid properties (table 1) indicates that conditions in the rumen are altered as a result of bloat. The far greater stability of foam which was artificially produced *in vitro* with rumen fluid samples drawn from bloated animals (nontreated), as compared with samples drawn from nonbloated animals (nontreated), apparently is a rather common development in cases of bloat. The increased surface tension, viscosity, and amount of foam and the decreased specific gravity are characteristic of the thick, foamy rumen ingesta which is usually present in bloated animals.

The decreased pH of rumen fluid from bloated animals may be due, at least in part, to carbon dioxide trapped in the sample. Perhaps pH is not a major factor in causing bloat, so long as normal pH

**TABLE 1—Influence of Detergent Treatment on Rumen Fluid Properties of Cattle Grazing Alfalfa**

| Cattle | No. of observations | IVI * (%) | Foam half life (min.) | Foam index (0–3) | Surface tension (dynes/cm.) | Viscosity (ratio) † | Specific gravity (ratio) † | pH |
|---|---|---|---|---|---|---|---|---|
| Bloated (nontreated) | 14 | 77 | 212 | 2.4 | 60.4 | 1.11 | 0.90 | 6.74 |
| Nonbloated (nontreated) | 20 | 32 | 63 | 0 | 55.1 | 1.02 | 0.99 | 7.09 |
| Detergent ‡ (nonbloated) (3 Gm./100 lb. body wt.) | 16 | 11 | 115 | 0.6 | 48.8 | 1.02 | 0.99 | 7.04 |
| Nontreated | 14 | 42 | 223 | 1.7 | 53.8 | 1.03 | 0.96 | 6.90 |

* Ingesta volume increase, an estimate of fermentation rate of rumen fluid *in vitro*. † Compared to an assigned value of 1.0 for water. ‡Ultrawet "K" (an alkyl aryl sodium sulfonate).

limits are not exceeded, although it may have an indirect role through its possible interrelationships with microbiological activity within the rumen and with the physical characteristics of the rumen ingesta. Whether bloat decreased as a result of the effect of the detergent on any one rumen fluid property, of the combined effect on several properties, or was unrelated to any effect on these properties is not known. However, a relationship between decreased bloat occurrence and rumen fluid properties as altered by the detergent is suggested by the data.

In general, bloat severity decreased in late summer. This could have been due to hot weather which reduced grazing or to poorer quality forage. Bloat occurrence did not appear to be closely related to atmospheric conditions, except that during uncomfortably hot periods grazing nearly ceased. In agreement with Johns,[9] at least mild bloating was observed in all stages of plant growth, including seed stage. It was observed that weeds and grasses were preferred, initially, to lush, young alfalfa. Not until the leaves were stripped from weeds and grasses did the cattle begin eating alfalfa extensively. Grazing habits were not altered noticeably as a result of daily administration of either the detergent or trisodium phosphate. The greatest incidence of bloat occurred in the early evening (approx. 6 to 9 p.m.) and the next greatest incidence in early morning (approx. 5 to 8 a.m.). That the majority of bloat cases occurred after 6 p.m. is in general agreement with observations made by Kephart,[10] but does not agree with those of Johns.[9]

EXPERIMENT 2—EFFECT OF A DETERGENT AND OF CHOLESTEROL WHEN ADDED TO ALFALFA JUICE

*Procedure.*—Fresh juice, obtained from chopped, green alfalfa by means of an Anderson oil expeller, was employed to produce bloat. Cholesterol was studied because of its known action in precipitating alfalfa saponins[16] and in inhibiting an alfalfa meal growth-depressing factor.[12] An alfalfa juice-cholesterol emulsion was prepared in a ball mill. Cholesterol, 2, 4, and 6 Gm. per pound of alfalfa juice (0.44, 0.88, and 1.32% of the total juice weight, respectively), was administered by stomach tube to dairy cattle ranging in age from 5 to 18 months. Fifteen different animals were employed for the 38 observations (table 2). Both sexes and several dairy breeds were employed. The only basis in the literature for dosage rates was the research conducted by Peterson,[12] wherein cholesterol successfully counteracted the growth-depressing factor in alfalfa meal when added to the diet of chicks at rates of 0.5 and 1.0 per cent of the ration.

The same detergent which was employed successfully as a bloat prophylactic in experiment 1 was employed in this experiment. Additions were made directly to the alfalfa juice preadministratively at the rate of either 30 or 60 Gm. per juice equivalent of 100 lb. of freshly chopped alfalfa. These rates were approximately equal to, and double, respectively, that employed with prophylactic success in the pasture grazing trial.

The animals were fed alfalfa hay and a concentrate mixture between bloat trials. Each animal was fasted for ten hours prior to administration of alfalfa juice, by stomach tube, to facilitate introduction of large amounts of juice and to standardize the degree of fill between animals. Surface tension, pH, foam half life, viscosity, and specific gravity of rumen fluid samples, from treated and control animals, were measured immediately prior to administration of alfalfa juice (equal to 8.5 to 10.0% of body weight) and at the time of maximum bloat (usually 3 to 5 hours after juice administration). In cases of no bloat, the samples were drawn between four and five hours postdrench. These five measurements were also made on the juice.

*Results and Discussion.*—Neither cholesterol nor the detergent, when added to alfalfa juice preadministratively at the various dosage levels, was effective in reducing bloat (table 2). Moreover, none

**TABLE 2—Effect of a Detergent and Cholesterol on the Severity of Bloat Induced by Alfalfa Juice**

| Treatment | No. of observations | Rate * | Av. bloat severity † |
|---|---|---|---|
| Control (juice alone) | 17 | .... | 2.1 |
| Detergent | 2 | 30 | 3.0 |
| | 5 | 60 | 3.1 |
| Cholesterol | 4 | 2 | 1.8 |
| | 8 | 4 | 2.4 |
| | 2 | 6 | 2.8 |

* Detergent, grams per juice equivalent of each 100 lb. of alfalfa forage; cholesterol, grams per pound alfalfa juice administered. Alfalfa juice was administered at levels of 8.5 to 10.0 per cent of body weight.

† Based on arbitrary values ranging from 0 (no bloat) to 5 (severe bloat).

of the measured properties of alfalfa juice or rumen fluid were greatly altered by addition of either product (table 3). The marked differences between pre- and postadministrative rumen fluid samples probably are due principally to the great dilution effect resulting from the large volume of plant juice administered.

tion of alfalfa juice, whereas it was quite effective against bloat resulting from grazing of alfalfa. The reason for this apparent contradiction is not clear; however, two different types of bloat may be involved. Certainly, the bloat resulting from alfalfa juice administration differed in many clinical respects and in character of the rumen ingesta (table 3) from pasture bloat (table 1). The negligible alteration of rumen fluid properties in the former case may be due to the absence of foam as contrasted to the foamy samples obtained from animals bloated on pasture.

## SUMMARY

Twenty-two beef cattle grazing alfalfa pasture were allotted to two groups and bloat-preventive treatments were alternated between groups. An alkyl aryl sodium sulfonate-type detergent (Ultrawet "K") was administered via gelatin cap-

**TABLE 3—Influence of a Detergent and of Cholesterol on Properties of Alfalfa Juice and Rumen Fluid**

| Treatment | No. of observations | Sample * source | Surface tension (dynes/cm.) | pH | Foam half life (min.) | Viscosity (ratio)† | Specific gravity (ratio)† |
|---|---|---|---|---|---|---|---|
| Control | 17 | plant | 45.8 | 5.73 | 16 | 1.09 | 1.06 |
| | | preadministration | 55.1 | 7.10 | 24 | 1.06 | 0.99 |
| | | postadministration | 48.7 | 5.68 | 74 | 1.05 | 0.99 |
| Detergent | 7 | plant | 40.1 | 5.78 | 31 | 1.14 | 1.04 |
| | | preadministration | 53.6 | 7.15 | 62 | 1.08 | 0.99 |
| | | postadministration | 42.4 | 5.60 | 47 | 1.05 | 1.00 |
| Cholesterol | 14 | plant | 46.7 | 5.65 | 38 | 1.08 | 1.06 |
| | | preadministration | 54.4 | 7.38 | 22 | 1.06 | 0.99 |
| | | postadministration | 47.0 | 5.75 | 65 | 1.05 | 1.00 |

* Plant (alfalfa) juice; preadministrative and postadministrative samples of rumen fluid. † Compared to an assigned value of 1.0 for water.

Apparently, the failure of cholesterol to prevent bloat was not due to method of administration, inasmuch as the aqueous emulsion, which was employed as a means of introducing the cholesterol into the extract to be administered, precipitated the cholesterol-precipitable saponins from alfalfa juice *in vitro*.[18] This could mean that *(a)* saponins are only partly (or not at all) involved etiologically in the alfalfa juice bloat syndrome; *(b)* saponins which are not precipitable with cholesterol are involved; *(c)* the saponin-cholesterol complex is dissociated within the rumen; or *(d)* the complex is bloat-provocative.

The detergent was essentially ineffective against bloat resulting from administra-

sules at rates of 20 and 30 Gm. daily per 1,000 lb. of body weight. Only at the higher rate did a significant reduction in incidence and severity of bloat occur. During bloat, the ingesta volume increase, foam durability, and degree of foaminess of rumen ingesta were markedly increased, surface tension and viscosity were moderately increased, while the specific gravity and pH were lowered moderately. All these properties were altered in the opposite direction (toward prebloat levels) during detergent treatment. Trisodium phosphate, 30 Gm. daily, had little influence on bloat occurrence or on the measured rumen fluid properties. Grazing habits were not altered

noticeably as a result of either detergent or trisodium phosphate treatment.

Bloat-provocative alfalfa juice was prepared and administered by stomach tube to young dairy animals. Neither cholesterol nor the detergent effectively reduced bloat or greatly altered the measured rumen fluid characteristics when added to the alfalfa juice before administration.

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# Studies of the Incidence and Morphology of Accessory Glandular Tissue in the Teat Canal of the Bovine Mammary Gland

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THERE ARE several detailed reports in the literature concerning the presence of functional accessory mammary secretory tissue in the walls of the lactiferous sinus and the teat sinus.[1,3,4] These structures have been termed "periductal lobules."[1]

This paper concerns some studies of the incidence and morphology of heretofore undescribed accessory glandular tissue structures in the wall of the teat canal (streak canal) of the dairy cow. These accessory glandular structures will be provisionally termed "periductal lobules."

## MATERIALS AND METHODS

While preparing tissue sections for classroom use, accessory glandular tissue was observed to be associated with the teat canal of a teat from a milking Holstein-Friesian cow. In addition, one other teat from this animal contained a bifid teat canal. Following these observations, 40 additional teats were obtained from the udders of 10 dairy cows at the local abattoir. The 40 specimens were fixed in calcium-cadmium formol and embedded in celloidin. Serial sections were cut at 30 $\mu$ in a plane which was parallel to the longitudinal axis of the teat. The sections were studied initially in an unstained and unmounted condition. It was found that this technique was sufficient for preliminary examinations because the epithelia appeared white in contrast to the surrounding tissues and, thus, were easy to see. Sample sections were then selected at intervals from the set of serial sections, stained with Weigerts' iron-hematoxylin and Crossman's Mallory triple stains,[2] and mounted on glass slides for further study.

## RESULTS AND DISCUSSION

One bifid teat canal (fig. 1) and one accessory gland in the streak canal (fig. 2) were observed in the four teats of the first udder studied. In the remaining 40 teats from ten additional udders, only one accessory periductal lobule was observed to originate from the wall of the teat canal (fig. 2).

The bifid teat canals appeared normal in structure and joined each other at the point of entry into the teat sinus. One was approximately one-third as large in diameter as the other, although both were functional.

The ducts of each of the two periductal lobules observed contained a stratified squamous epithelium and each originated from the teat canal at an acute angle at a level approximately three-fourths of the distance from the apex of the teat (fig. 2 and 3) to the rosette of Fürstenberg. Both

**Fig. 1—An oblique section through the tip of teat (A) of cow 1 showing bifid teat canal: (a) apex of teat; (b) smaller teat canal; (c) larger teat canal; (d) level of rosette of Fürstenberg. x 9.5.**

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Fig. 2—A longitudinal section through the tip of teat (B) of cow 1 showing the periductal lobule in longitudinal section. The section is cut immediately lateral to the lumen of the teat canal: (a) junction of teat canal and teat sinus; (b) glandular portion of periductal lobule. x 9.5.

Fig. 3—An oblique section through the teat canal of cow 2 near the level of the teat canal-teat sinus junction (a), showing the origin (b) of the duct of the periductal lobule from the teat canal. x 9.5.

ducts were patent throughout and terminated in a small lobule of glandular tissue which appeared to be functional. The dorsal extremity of one lobule (fig. 1) extended slightly above the level of the rosette of Fürstenberg. The duct of the other was considerably longer and somewhat larger. The dorsal extremity of this lobule was approximately 10 mm. above the level of the rosette.

Examinations of the glandular portions of the periductal lobules disclosed a central saccular duct with short, radially directed ducts extending from it (fig. 4). The latter terminated as tubular structures or showed slight alveolar dilatations. The epithelium of the central saccular portion was of the characteristic two-cell-layer-type seen in the lactiferous and teat sinuses and in the larger ducts in the mammary gland (fig. 5). The epithelia of the ducts and terminal alveolar dilatations consisted of a single layer of columnar cells which measured approximately 15 $\mu$ in height.

The lumen of the central saccular duct contained a considerable amount of milk-protein precipitate. Many agranulocytes of the small lymphocyte type, together with considerable numbers of heterophils and a few eosinophils, were embedded in these lumen contents. Occasional large cells,

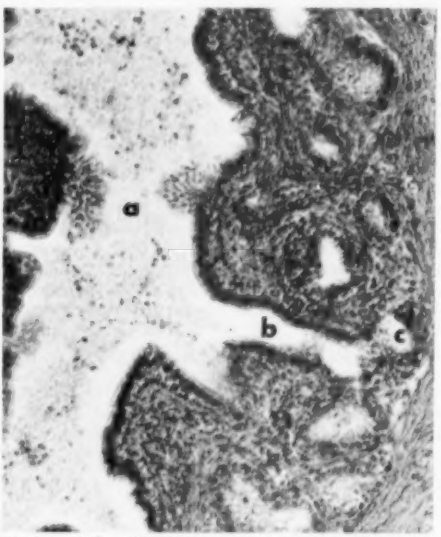


Fig. 4—A longitudinal section through the glandular portion of the periductular lobule of the teat of cow 1. Note the precipitated secretory material, leukocytes, and epithelial cells in the lumen of the central saccular portion (a) of the lobule; (b) radially directed duct; (c) terminal alveolar dilatation. x 275.

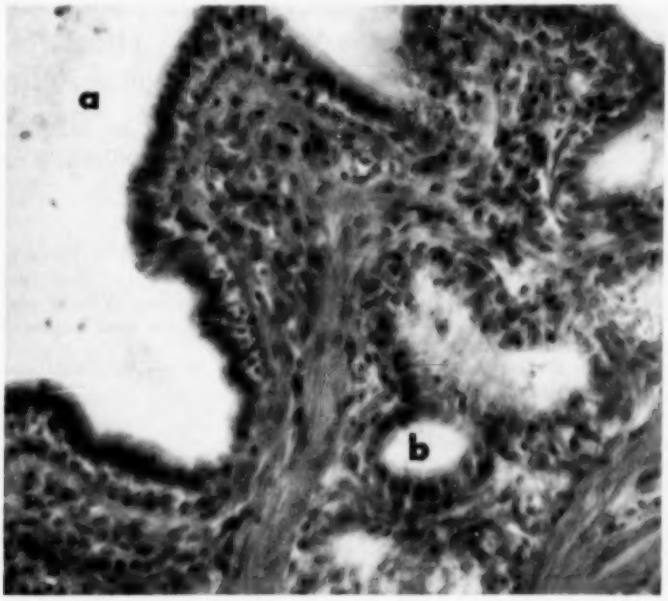


**Fig. 5—A longitudinal section of a portion of a periductal lobule showing the two-layered epithelium of the central saccular duct (a) and the simple columnar epithelium of the terminal alveolar structure (b). x 440.**

which appeared to be sloughed epithelial cells, were also present. The main duct, which extended from the lobule to the teat canal, was free of cellular material and other microscopically visible noncellular elements. These findings were interpreted as indicating that the glandular elements of the periductal lobules were secreting, though at a very slow rate.

## Summary and Conclusions

1) Serial celloidin sections were made of the teats of 11 dairy cows which were slaughtered at a local abattoir. The sections were examined for the presence of accessory glandular tissue which was associated with the teat canal. These structures were termed "periductal lobules."

2) Only two teats from 11 cows studied contained these accessory glandular structures. One other teat contained a bifid teat canal.

3) The main duct of the periductal lobules arose from the teat canal at an acute angle and in a position which was approximately three-fourths of the distance from the apex of the teat to the rosette of Fürstenberg. The epithelium of the main duct was stratified squamous in type. In the central saccular portion, it was of the characteristic two-layer type. The terminal secretory ducts and alveoli contained a single-layered, low columnar epithelium.

4) The presence of a milk-protein precipitate with a considerable accumulation of leukocytes indicated that the glandular tissue was secreting, though probably at a very slow rate.

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# The Characteristics of Agglutinating Antigens of *Vibrio Fetus* Variants. I. Effects of Heat and Formalin on Serological Activity

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An analysis of serological studies of *Vibrio fetus* infected cattle indicates that the bacterial agglutination test has not been found entirely satisfactory as a diagnostic tool in such infections or in clarifying the antigenic relationships between strains of diverse origin. In previous studies, Ristic *et al.*[1] indicated that variation of the S—R variety could account for heterogeneity within strains, *i.e.*, dissociation to nonsmooth forms resulted in the loss of cross-reactivity with antiserums prepared against smooth forms.

It has been observed in some organisms that superficial heat-labile antigens serve to mask deep antigens and so confuse the serological relationship, e.g. the L antigen of *Escherichia coli* and the Vi antigen of typhoid bacillus.[2,3] The effects of heat on antigenicity of *V. fetus* have been reported by Wiidik and Hlidar[6] who found that heat treatment served to change the antigenicity of the organism, from which evidence they postulated the existence of a heat-labile "K" antigen. However, their experiments did not include control of colonial and concomitant antigenic variational phenomena. Thus, the effects of heating on the antigenicity of whole cell *V. fetus* antigens were also studied with a view to observing the cross-reactivity between strains and variants of strains before and after such treatment.

The present report extends serological studies of colonial variants of *V. fetus* to include freshly isolated strains of bovine, ovine, and human origin and a recently isolated "saprophytic," noncatalase-producing Vibrio in regard to both heat-labile and heat-stable antigens.

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## Materials and Methods

Five of the strains studied (53-D, I, UF-1, F, and 1134) were selected as representative of *V. fetus* on the basis of their cultural, morphological, and biochemical characteristics. Strains 53-D and I were isolated from aborted ovine fetuses and strains UF-1 and F originated from an aborted bovine fetus and the cervical mucus of a cow of low fertility, respectively. Strain 1134 was isolated from a human patient with subacute bacterial endocarditis. Strains 53-D, UF-1, and 1134 had been maintained in the laboratory for periods of one to three years in Brucella-Albimi * broth to which 0.16 per cent agar had been added. Strains I and F were lyophilized immediately upon isolation and reactivated for use in this experiment. In addition to the above "true" *V. fetus* strains, a noncatalase-producing "saprophytic" Vibrio, strain 4220, was also used in this study. This strain had been recently isolated from the vaginal mucosa of an apparently normal fertile cow. Colonial variants were selected as described in an earlier report.[4] The variant previously designated "very rough" is referred to as "rough" in this report.

Agglutinating antigens consisted of washed cells obtained from 72-hour growth on Brucella-Albimi agar to which 0.5 per cent agar was added, which resulted in a final agar concentration of 2.0 per cent. Formolized antigens were prepared by suspending the cells in 0.3 per cent formaldehyde solution containing 0.85 per cent sodium chloride. Heat-treated antigens were prepared by boiling washed cells for two hours, washing twice by centrifugation, and finally suspending the cells in 0.3 per cent formaldehyde-saline solution. The concentrated antigens were stored in the refrigerator. Prior to use, the antigens were standardized to a density of approximately 55 per cent light transmission in formaldehyde-saline solution using a Leitz photoelectric colorimeter measured at 550 mμ. The density approximated tube three of McFarland's nephelometer. Antiserums were prepared by inoculating rabbits intravenously four times during a 21-day period with dense formalin-

* A product of Albimi Laboratories, Brooklyn, N. Y.

killed suspensions (approximately $10^9$ cells per ml.) of the above described respective antigens.

Agglutination tests were conducted by preparing a 1:5 dilution of the specific antiserum in formaldehyde-saline solution. Subsequent dilutions consisted of serial transfers of 0.5-ml. amounts of the diluted antiserum in 0.5 ml. formaldehyde-saline solution. Antigen in the amount of 0.5 ml. was added to make final dilutions of 1:10, 1:20, 1:40, et cetera. The mixtures were incubated at 37 C. for 24 hours before results were recorded. The titer of the serums was expressed in terms of the highest dilution of serum giving complete agglutination. The criterion for complete agglutination was evidence of a clear supernatant fluid with presence of clumps when tubes were gently agitated.

In agglutinin-absorption tests, absorbing capacity of the antigens for antiserums was first determined. This was done by adding 0.5 ml. of a given antiserum to each of four tubes containing 4.5-ml. amounts of the antigen standardized to a density of 1, 2, 4, and 6, respectively, on the McFarland scale. The tubes were incubated for three hours at 37 C., after which time they were centrifuged and the supernatant fluids tested for agglutinins.

## Results

Cross-agglutination reactions among formolized smooth and rough cells and their antiserums are shown (table 1). Various degrees of heterogeneity, and in one case (strain I) complete inagglutinability, were shown between smooth and rough variants within the parent strain. Antigenic similarity was demonstrated between smooth antigens of two ovine strains, 53-D (Colorado) and I (Illinois), while the smooth cells of two strains of bovine origin recovered in Florida were serologically heterologous. Antiserum prepared against smooth cells of strain F agglutinated the smooth antigen 1134, of human origin, in a serum dilution of 1:2,560. In general, strains 53-D, I, and UF were related on the basis of their smooth antigens while, on the same basis, strains 1134 and F appeared to be related to each other. However, reciprocal relationships did not always hold, e.g. UF antibody vs. I antigen indicated no relationship while the I antibody vs. UF antigen did indicate a relationship. This also held for strains 1134 and F.

On the basis of rough antigens, this grouping was again evident except that the F-1134 relationship no longer held, and that increased cross-reactivity was seen between F antiserum and 53-D and I antigens, and I antiserum and F antigen. Also, the reciprocal relationship between 53-D and I which held with smooth antigen no longer held with rough antigens.

When antiserums prepared against S cells were reacted with R antigens, new relationships appeared (e.g. 1134 antiserum vs. F antigen), and some were lost (e.g., I antiserum vs. all antigens including homologous strains).

Data pertaining to cross-agglutination reactions of formolized and heated antigens and their antiserums are summarized (table 2, A-H). In every case where a cross-reaction occurred between strains on the basis of their formolized antigen, essentially the same degree of cross-reaction was observed when heated antigen was employed (table 2, A). However, in four instances (1134 vs. 53-D antiserum, 1134 vs. I antiserum, I vs. UF antiserum, and F vs. 1134 antiserum), cross-reaction was observed with heated antigen where the formolized antigen had failed to agglutinate. In two instances, the titer against heated antigen was three serial dilutions higher

**TABLE I—Cross-Agglutination Reactions Among Formolized Smooth and Rough Vibro Fetus Antigens and Their Antiserums**

| Strain origin | Antigen | | Antiserum: 53-D S | 53-D R | I S | I R | UF-1 S | UF-1 R | F S | F R | 1134 S | 1134 R |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ovine | 53-D | S | 5,120* | 1,280 | 320 | 40 | 2,560 | 1,280 | 40 | 0 | 0 | 0 |
| | | R | 640 | 82,000 | 0 | 160 | 20,480 | 10,240 | 80 | 80 | 320 | 10 |
| | I | S | 320 | 0 | 5,120 | 320 | 0 | 0 | 20 | 40 | 0 | 0 |
| | | R | 320 | 0 | 0 | 1,280 | 0 | 0 | 160 | 1,280 | 80 | 0 |
| Bovine | UF-1 | S | 640 | 5,120 | 640 | 40 | 41,000 | 2,560 | 40 | 20 | 40 | 0 |
| | | R | 160 | 2,560 | 40 | 160 | 1,280 | 82,000 | 20 | 40 | 40 | 0 |
| | F | S | 0 | 0 | 0 | 0 | 0 | 0 | 10,240 | 320 | 0 | 0 |
| | | R | 640 | 0 | 0 | 640 | 80 | 0 | 160 | 10,240 | 80 | 0 |
| Human | 1134 | S | 0 | 80 | 0 | 0 | 40 | 40 | 2,560 | 80 | 2,560 | 1,280 |
| | | R | 20 | 10 | 0 | 0 | 0 | 20 | 40 | 0 | 160 | 2,560 |

* Titer expressed as reciprocal of serum dilution. S = smooth variant cells; R = rough variant cells.

**TABLE 2—Cross-Agglutination Reactions Among Variant Antigens and Antiserums of Five Vibrio Fetus Strains**

| A Smooth antigens | | Antiserums prepared against smooth *formolized* cells | | | | |
|---|---|---|---|---|---|---|
| | | 53-D | I | UF-1 | F | 1134 |
| 53-D | F | 5,120* | 320 | 2,560 | 40 | 0 |
| | H | 1,280 | 640 | 0 | 0 | 0 |
| I | F | 320 | 5,120 | 0 | 20 | 0 |
| | H | 640 | 640 | 80 | 0 | 0 |
| UF-1 | F | 640 | 640 | 41,000 | 40 | 40 |
| | H | 2,560 | 2,560 | 2,560 | 0 | 160 |
| F | F | 0 | 0 | 0 | 10,240 | 0 |
| | H | 0 | 0 | 0 | 10,240 | 2,560 |
| 1134 | F | 0 | 0 | 40 | 2,560 | 2,560 |
| | H | 80 | 80 | 640 | 20,480 | 2,560 |

| B Smooth antigens | | Antiserums prepared against smooth *heated* cells | | | | |
|---|---|---|---|---|---|---|
| | | 53-D | I | UF-1 | F | 1134 |
| 53-D | F | 0 | 0 | 0 | 0 | 0 |
| | H | 2,560* | 0 | 0 | 0 | 2,560 |
| I | F | 0 | 0 | 0 | 0 | 0 |
| | H | 320 | 640 | 0 | 0 | 0 |
| UF-1 | F | 0 | 0 | 0 | 0 | 0 |
| | H | 2,560 | 2,560 | 5,120 | 0 | 2,560 |
| F | F | 0 | 0 | 0 | 0 | 0 |
| | H | 0 | 0 | 0 | 10,240 | 1,280 |
| 1134 | F | 0 | 0 | 0 | 0 | 0 |
| | H | 0 | 0 | 320 | 10,240 | 10,240 |

| C Rough antigens | | Antiserums prepared against rough *formolized* cells | | | | |
|---|---|---|---|---|---|---|
| | | 53-D | I | UF-1 | F | 1134 |
| 53-D | F | 82,000* | 160 | 10,240 | 80 | 10 |
| | H | 10,240 | 2,560 | 0 | 320 | 0 |
| I | F | 0 | 1,280 | 0 | 1,280 | 0 |
| | H | 10,240 | 2,560 | 2,560 | 1,280 | 320 |
| UF-1 | F | 2,560 | 160 | 82,000 | 40 | 0 |
| | H | 5,120 | 1,280 | 10,240 | 2,560 | 1,280 |
| F | F | 0 | 640 | 0 | 10,240 | 0 |
| | H | 80 | 160 | 640 | 2,560 | 1,280 |
| 1134 | F | 10 | 0 | 20 | 0 | 2,560 |
| | H | 80 | 640 | 10,240 | 20 | 1,280 |

| D Rough antigens | | Antiserums prepared against rough *heated* cells | | | | |
|---|---|---|---|---|---|---|
| | | 53-D | I | UF-1 | F | 1134 |
| 53-D | F | 0 | 0 | 0 | 0 | 0 |
| | H | 1,280* | 640 | 0 | 2,560 | 640 |
| I | F | 0 | 0 | 0 | 0 | 0 |
| | H | 640 | 1,280 | 640 | 1,280 | 160 |
| UF-1 | F | 0 | 0 | 0 | 0 | 0 |
| | H | 640 | 1,280 | 5,120 | 2,560 | 640 |
| F | F | 0 | 0 | 0 | 0 | 0 |
| | H | 160 | 160 | 1,280 | 1,280 | 320 |
| 1134 | F | 0 | 0 | 0 | 0 | 0 |
| | H | 1,280 | 1,280 | 1,280 | 1,280 | 640 |

| E Smooth antigens | | Antiserums prepared against rough *formolized* cells | | | | |
|---|---|---|---|---|---|---|
| | | 53-D | I | UF-1 | F | 1134 |
| 53-D | F | 1,280* | 40 | 1,280 | 0 | 0 |
| | H | 1,280 | 0 | 0 | 0 | 0 |
| I | F | 0 | 320 | 0 | 40 | 0 |
| | H | 160 | 0 | 0 | 320 | 0 |
| UF-1 | F | 5,120 | 40 | 2,560 | 20 | 0 |
| | H | 10,240 | 640 | 10,240 | 40 | 0 |
| F | F | 0 | 0 | 0 | 320 | 0 |
| | H | 0 | 320 | 0 | 320 | 160 |
| 1134 | F | 80 | 0 | 40 | 20 | 1,280 |
| | H | 1,280 | 640 | 5,120 | 320 | 640 |

| F Smooth antigens | | Antiserums prepared against rough *heated* cells | | | | |
|---|---|---|---|---|---|---|
| | | 53-D | I | UF-1 | F | 1134 |
| 53-D | F | 0 | 0 | 0 | 0 | 0 |
| | H | 640* | 160 | 0 | 0 | 0 |
| I | F | 0 | 0 | 0 | 0 | 0 |
| | H | 640 | 0 | 320 | 20 | 0 |
| UF-1 | F | 0 | 0 | 0 | 0 | 0 |
| | H | 640 | 160 | 2,560 | 2,560 | 320 |
| F | F | 0 | 0 | 0 | 0 | 0 |
| | H | 80 | 160 | 0 | 1,280 | 40 |
| 1134 | F | 0 | 0 | 0 | 0 | 0 |
| | H | 640 | 320 | 320 | 1,280 | 160 |

| G Rough antigens | | Antiserums prepared against smooth *formolized* cells | | | | |
|---|---|---|---|---|---|---|
| | | 53-D | I | UF-1 | F | 1134 |
| 53-D | F | 640* | 0 | 20,480 | 80 | 320 |
| | H | 5,120 | 2,560 | 0 | 80 | 640 |
| I | F | 320 | 0 | 0 | 160 | 80 |
| | H | 320 | 40 | 640 | 160 | 320 |
| UF-1 | F | 160 | 40 | 1,280 | 20 | 40 |
| | H | 5,120 | 1,280 | 1,280 | 80 | 320 |
| F | F | 640 | 0 | 80 | 160 | 80 |
| | H | 160 | 20 | 320 | 1,280 | 80 |
| 1134 | F | 20 | 0 | 0 | 40 | 160 |
| | H | 160 | 80 | 2,560 | 1,280 | 2,560 |

| H Rough antigens | | Antiserums prepared against smooth *heated* cells | | | | |
|---|---|---|---|---|---|---|
| | | 53-D | I | UF-1 | F | 1134 |
| 53-D | F | 0 | 0 | 0 | 0 | 0 |
| | H | 5,120* | 10,240 | 40 | 20 | 160 |
| I | F | 0 | 0 | 0 | 0 | 0 |
| | H | 20 | 1,280 | 160 | 0 | 40 |
| UF-1 | F | 0 | 0 | 0 | 0 | 0 |
| | H | 5,120 | 80 | 640 | 20 | 80 |
| F | F | 0 | 0 | 0 | 0 | 0 |
| | H | 0 | 20 | 0 | 2,560 | 80 |
| 1134 | F | 0 | 0 | 0 | 0 | 0 |
| | H | 20 | 20 | 80 | 640 | 640 |

* Titer expressed as reciprocal of serum dilution. F = formolized antigen; H = antigen heated for two hours by boiling.

than that exhibited by formolized antigen (1134 vs. UF and F antiserums). Other cases indicated a slightly lower titer when heated antigen was employed, but such differences were not considered significant when they were less than three serial dilutions.

The results obtained when antiserums prepared against smooth heated cells were tested with two antigen preparations are illustrated (table 2, B). In no instance were formolized antigens agglutinated even when homologous strain antiserums were employed. The cross-reactions between heterologous strains when heated antigens were employed were essentially the same as those exhibited (table 2, A), with the exception of a new cross-reaction which was exhibited by strain 53-D vs. 1134 anti-

serum. When rough antigens were tested with antiserums prepared against rough formolized cells (table 2, C), heated antigens exhibited a significantly higher† titer in 13 tests, a significant drop in titer in three tests with a degree of cross-reaction existing among almost all strains. Rough formolized antigens did not react with antiserums prepared against rough heated cells (table 2, D) as observed previously with smooth antigens (table 2, B).

With the exception of one test (table 2, D, 53-D vs. UF antiserum) in which no cross-reaction was shown, all tests revealed considerable degrees of cross-reaction. In seven instances, smooth antigens, both heated and formolized, tested with antiserums prepared against rough formolized cells (table 2, E) demonstrated no cross-reaction. With heated antigens, the titer was increased significantly in nine tests and reduced in two tests. Again there was no cross-reaction between smooth formolized antigens and antiserums prepared against rough heated cells (table 2, F). However, antiserums of rough heated cells reacted to a considerable degree with smooth heated antigens with the exception of six tests in which no demonstrable reactions occurred. Rough antigens agglutinated with antiserums of smooth formolized cells (table 2, G), and demonstrated cross-reaction in all tests. In 12 instances, heated antigens were agglutinated in higher dilutions of antiserums than were nonheated antigens. Rough formolized antigens were inagglutinable with antiserums of smooth heated cells (table 2, H). On the other hand, rough heated antigens showed slight degrees of cross-reaction among the strains with the exception of three tests in which no cross-reaction was demonstrated.

† A difference between titers was considered significant only if it was a matter of three serial dilutions or more, the criterion which is used in all subsequent discussions.

**TABLE 3—Summary of Results of Cross-Agglutination Reactions Among Variant Antigens and Antiserums of Five Vibro Fetus Strains**

| Antigens | Antiserums prepared against S-F | S-H | R-F | R-H |
|---|---|---|---|---|
| S-F | 6* | 0 | 3 | 0 |
| S-H | 11 | 8 | 10 | 13 |
| R-F | 10 | 0 | 7 | 0 |
| R-H | 18 | 9 | 17 | 19 |

Agglutination reactions in serum dilutions of 1:80 and higher were regarded as positive tests.

* Number of tests positive out of a total of 20 possible combinations.

S = smooth variant cells; R = rough variant cells; F = formolized cells; H = heat-treated cells.

Summary of results of cross-agglutinations among antigens and variants of *V. fetus* strains based on reactions in serum dilutions of 1:80 and higher are shown (table 3). Of a total of 20 possible heterologous cross-reactions in each combination of tests, the highest number of cross-reactions resulted when rough heated antigens were tested with antiserums prepared against rough heated cells. Only three cross-reactions were observed when smooth formolized antigens were agglutinated with antiserums prepared against rough formolized cells. In no instance did reaction occur when formolized antigens were tested

**TABLE 4—Cross-Agglutination Reactions Between the Antigens of Noncatalase Producing Vibrio Strain 4220 and Antiserums Prepared Against the Smooth and Rough Antigens of Five Vibrio Fetus Strains**

| Antigens 4220 * | 53-D | I | UF-1 | F | 1134 | 4220 * |
|---|---|---|---|---|---|---|
| | Antiserums prepared against smooth *formolized* cells | | | | | |
| F | 0 | 0 | 0 | 0 | 0 | 5,120 |
| H | 40† | 10 | 80 | 20 | 20 | 320 |
| | Antiserums prepared against smooth *heated* cells | | | | | |
| F | 0(320) | 0(320) | 0(320) | 0(80) | 0(160) | 160 |
| H | 80(160) | 20 | 160(160) | 40 | 80(160) | 2,560 |
| | Antiserums prepared against rough *formolized* cells | | | | | |
| F | 0 | 20 | 40 | 80 | 0 | 5,120 |
| H | 160 | 80 | 40 | 80 | 40 | 320 |
| | Antiserums prepared against rough *heated* cells | | | | | |
| F | 0 | 20 | 0(640) | 0(40) | 0 | 160 |
| H | 80 | 80 | 160(320) | 80 | 80 | 2,560 |

Figures in parentheses represent the reciprocal reactions, i.e., reversal of the antigen and antiserum used.

* Antigen in "cut glass" variant type. † Antiserum prepared against "cut glass" variant type. ‡ Titer expressed as reciprocal of serum dilution.

F = formolized antigen; H = antigen heated for two hours by boiling.

with antiserums prepared against heated cells.

Results of agglutination reactions between "saprophytic" Vibrio strain 4220 and *V. fetus* strains are shown (table 4). Heated antigens of strain 4220 reacted in serum dilutions of 1:20 to 1:160 with antiserums prepared against both variants of the five *V. fetus* strains. Formolized antigen of strain 4220 reacted in dilutions of 1:20 to 1:80 only with antiserums of rough formolized cells of three *V. fetus* strains. Heated antigens of smooth *V. fetus* variants reacted with antiserums of strain 4220 in eight tests in serum dilutions of 1:80 to 1:320. Heated antigens of rough *V. fetus* variants reacted in three tests with antiserums of strain 4220. Formolized antigen of strain 4220 reacted with antiserum prepared against heated cells of the homologous strain in a dilution of 1:160, whereas such antiserums in the case of "true" *V. fetus* failed to exhibit any reaction with formolized antigen.

Agglutination results obtained following absorption of antiserums prepared against formolized cells with heated antigens are shown (table 5). Antiserums prepared against smooth formolized cells contained agglutinins which reacted with both heated and formolized antigens. The agglutinins reacting with heated antigen were absorbed completely with heated cells, leaving essentially unabsorbed the agglutinins against formolized cells. Rough heated cells, however, removed practically equal amounts of agglutinins which had reacted with both heated and formolized antigens.

## DISCUSSION

The existence of a thermolabile superficial antigen of the smoth *V. fetus* cell was demonstrated by: (1) inagglutinability of the formolized cell with antiserums prepared against the heat-treated cell, and (2) absorption of homologous agglutinins by heated cells from the serums prepared against formolized antigen without effect on the agglutinating titer of this antigen.

The heat-stable component does not appear to be an artifact due to heating the antigen. Antiserums which had been produced with cells never exposed to a high degree of heat sometimes reacted with heated antigens to an even higher titer than with unheated antigens. That such heat-stable antigens are not located superficially on the cell is indicated by the finding that heating, in removing or destroying the superficial heat-labile component, increases cross-reactivity between strains.

Rough antigens, on the other hand, did not follow the agglutinin-absorption pattern of a smooth cell. When rough heated cells were employed to absorb the serums prepared against rough formolized cells, the agglutinating titers of both heated and formolized antigens were reduced. This indicates that the heat-labile portion of a rough cell does not prove analogous to the smooth cell which represents a complete, antigenically distinguishable cell envelope, as demonstrated in the agglutinin-absorption test. It is apparent from these data that serological differences between intact cells of smooth and rough variants may have resulted from the quantitative differences of their heat-labile antigens. However the process of S—R mutation could not be limited entirely to degradation of the superficial antigen, since a degree of serological heterogeneity was demonstrated among heat-treated antigens of both variants. Degradation of the superficial antigen could conceivably be responsible for the spontaneous agglutination of nonsmooth variants in saline solution and normal bovine serum.[5] Loss of the super-

**TABLE 5—Reactions of Antiserums Prepared Against Formolized Cells Before and After Absorption with Heated Antigens**

| Strain origin | Antiserum | Absorbed by | Antigen | Serum titer | | No. of dilutions drop in titer |
|---|---|---|---|---|---|---|
| | | | | Unabsorbed | Absorbed | |
| I Ovine | S-F | S-H | S-F | 82,000* | 10,000 | 3 |
| | | | S-H | 10,000 | 0 | 11 |
| | R-F | R-H | R-F | 82,000 | 80 | 10 |
| | | | R-H | 324,000 | 160 | 11 |
| F Bovine | S-F | S-H | S-F | 82,000 | 20,000 | 2 |
| | | | S-H | 20,000 | 0 | 12 |
| | R-F | R-H | R-F | 41,000 | 1,280 | 5 |
| | | | R-H | 10,000 | 320 | 5 |

* Titer expressed as reciprocal of serum dilution. S = smooth variant cells; R = rough variant cells; F = formolized cells; H = heat-treated cells.

ficial antigen may have instigated an electric change on the bacterial cell with the consequence that the potential fell below the critical value, which produced a decrease of suspension stability in weak electrolytes and resulted in spontaneous agglutination.

In evaluating the structural differences between smooth and rough cells on the basis of their agglutinin-absorption pattern, the existence of a third or flagellar antigen of *V. fetus* cell should not be overlooked. In fact, the previous report showed the rough cells to be considerably less motile.[4] This indicates necessity for a detailed study on structural configuration of the entire *V. fetus* cell of colonial variants in order that the cellular mutation phenomenon and its accompanying antigenic alteration may be clearly understood.

Data presented (table 2) suggest that serological heterogeneity of the variants within the parent strain and among the heterologous strains is considerably minimized by use of heat-treated antigens. In general, heated antigens, when compared with formolized antigens, demonstrated a titer increase from one to 12 serial serum dilutions in cross-agglutination tests. Practical importance of the data presented, however, could only be evaluated by conducting similar studies using serums from cattle which were naturally infected. In so doing, the serological spectrum of the two antigenic preparations could be evaluated, which might lead to the preparation of a test antigen of broader serological activity. Such studies are in progress.

The lack of a reciprocal relationship between antigen and antiserum in some cases would seem to indicate that some strains may share antigens, but that the sharing may not be apparent in the agglutination reaction. That is, an antigen, if it is not a surface component, or if it is a quantitatively minor component, would not react in the agglutination test when used as a cell antigen, but would act antigenically, inducing the production of agglutinins in a rabbit. Hence, relationships between strains may become apparent only when the reciprocal reaction tests are performed (e.g., UF vs. I, table 2, A and 53-D vs. I, table 2, B).

Strain 4220, being catalase-negative and $H_2S$-positive, is presently characterized as a "saprophytic" Vibrio strain which inhabits the bovine genital mucosa.[1] Colonial dissociative phenomena of this strain greatly differ from those of catalase-producing *V. fetus* strains.[5] On the other hand, heat-stable antigens of *V. fetus* variants were, to a degree, shared serologically with strain 4220. Should noncatalase-producing Vibrio prove nonpathogenic for cattle, it would be of interest to examine the immunological status of a host this organism inhabits in respect to: (1) resistance to infection with *V. fetus*, and (2) presence of antibodies reactive with *V. fetus* antigens.

Strain 1134, of human origin, on the basis of its serological reactivity and biochemical and cultural characteristics, including colonial dissociation phenomena, was not distinguishable from a *V. fetus* of bovine origin (e.g. strain F). Whether strain 1134 is strictly a human *V. fetus*, or whether such a designation means only priority in isolation, is a subject for further research.

## Summary

Presence of a thermolabile superficial antigen on the smooth *Vibrio fetus* cell was demonstrated by use of agglutination and agglutinin-absorption tests. This characteristic was not completely substantiated on the rough variant cell in the agglutinin-absorption test. Boiling for two hours destroyed or removed completely the superficial antigen of "true" *V. fetus* and only partially degraded or removed the superficial antigen of the "saprophytic" Vibrio.

Degrees of heterogeneity and inagglutinability were demonstrated between smooth and rough variants within the parent culture and among the variants of five strains. Serological heterogeneity of the variants was considerably minimized by use of heat-treated antigens. The greatest number of cross-reactions between strains resulted when rough heated antigens were agglutinated with antiserums prepared against rough heated cells. Heat stable antigens of *V. fetus* variants were, to a degree, serologically shared with "saprophytic" noncatalase-producing Vibrio.

On the basis of the data presented, it appears that use of heated antigen in addition to formolized antigen may aid in the interpretation of the serological tests used for the diagnosis of vibriosis.

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# Immunization of Cattle Against Experimental Anthrax with Alum-Precipitated Protective Antigen or Spore Vaccine

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ELABORATION of the protective antigen of *Bacillus anthracis, in vitro,* was first demonstrated by Gladstone.[4] Chemically defined, nonprotein mediums for production of the antigen were developed by Wright *et al.*[8, 13] Protective antigen present in filtrates of the cultures was concentrated and stabilized by precipitation with alum. Immunizing activity of the alum-precipitated antigen was demonstrated by cutaneous challenge in rabbits and guinea pigs, and by cutaneous and respiratory challenge in monkeys.[12] The antigen protected rabbits against challenge with strains of *B. anthracis* of various origins.[1] Properties of similar preparations of antigen, produced in more complex mediums, have been described by Belton and Strange,[2] and by Boor.[3]

Immunization of cattle, sheep, and swine with the alum-precipitated antigen was studied by Schlingman *et al.*[9] They developed an oral challenge procedure that produced fatal infection in a large proportion of normal cattle. Two injections of alum-precipitated protective antigen produced significant immunity against this challenge procedure. Sheep resisted intracutaneous injection of 200 $l.d._{50}$ or more of spores three months after immunization; the immunity had declined appreciably after five to seven months.

Further experiments with larger numbers of cattle seemed necessary for estimation of the usefulness of the product under field conditions. It was of interest to determine whether a single injection of antigen would produce satisfactory immunity. Preliminary comparison of the protection afforded by the sterile antigen with that produced by an improved spore vaccine of the type described by Sterne[10] was also undertaken, and certain features of the experimental infection were studied.

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## MATERIALS AND METHODS

*Antigen Preparations.*—Two preparations of alum-precipitated protective antigen were used. Both were produced by growth of the RI-NP strain of *B. anthracis* in 599-medium.* The RI-NP strain is a nonproteolytic, nonencapsulated mutant of the Vollum strain. The sterile, antigen-containing culture filtrates were precipitated by addition of 0.1 per cent aluminum potassium sulfate followed by adjustment to pH 5.9. The precipitates were washed and resuspended in saline solution. Preparations 690 and 761 were concentrated tenfold and twenty-fold, respectively, in terms of the original culture filtrate. Merthiolate at a concentration of 1:10,000 was added as a preservative. Sterility was established by culture of the original filtrates and the alum-precipitated products, and by injection of the latter into guinea pigs and mice, as described previously.[8, 12]

The antigenic activity of the preparations was tested by immunization of rabbits.[12] Dilutions of the stock antigen were prepared in saline solution and groups of rabbits were immunized by intracutaneous injection of five 0.5-ml. doses on alternate days. The dilutions were doubled for preparation 761 because this product had been concentrated twenty-fold from the original filtrate. Seven days after immunization, the animals were challenged by the intracutaneous injection of 10,000 spores of the Vollum strain of *B. anthracis.* Each preparation was assayed in rabbits before and after it was used for immunization of cattle. The results of the successive assays were not significantly different (table 1). The preparations were similar in antigenic activity to those used in previous investigations.[12]

*Spore Vaccine.*—The anthrax spore vaccine was Carbozoo R nonencapsulated.*

*Challenge Suspension for Cattle.*—The V770-2-P strain of *B. anthracis* was used. This strain had been isolated from cattle with anthrax in Florida in 1951, and had been passaged in a cow and then in a guinea pig.[5] The spore suspension had been prepared by growth of the strain on nutrient agar; the growth was washed off in water and heated at 56 C. for 30 minutes. The spore suspension used by Schlingman *et al.*[9] was employed. The initial viable count on the suspension was

* Provided by Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

$1.5 \times 10^8$ spores per milliliter; this decreased gradually during the course of the work to $6.5 \times 10^7$. Viable counts were determined before each group of animals was challenged, and the volume of the challenge dose was adjusted so that $1.5 \times 10^8$ spores were given in all cases.

*Animals.*—Hereford steers, approximately 18 months old and weighing 500 to 700 lb., were used. They had been raised in an area of Texas generally reputed to be free of anthrax. The steers were marked for identification and were kept together under ideal range conditions with supplementary feedings as required. One week before challenge, they were trucked 135 miles to the challenge area.

**TABLE 1—Assay of Antigen in Rabbits**

| Antigen preparation | Dilution of antigen | Rabbits surviving / Rabbits challenged |
|---|---|---|
| 690 | 1:10 | 4/6 |
| | 1:30 | 6/6 |
| | 1:90 | 4/6 |
| 761 | 1:20 | 6/6 |
| | 1:60 | 6/6 |
| | 1:180 | 2/6 |

The animals were challenged in a screened isolation barn equipped with a concrete floor and a closed drainage system. Survivors were held at least 21 days after challenge. All that died of anthrax, and those that were killed, were necropsied as soon as possible and completely cremated within 24 hours.

*Route and Method of Challenge.*—All steers were challenged by the oral route.[8] Commercial cattle feed pellets were selected for length and compactness. Usually, pellets approximately 3 inches long could be obtained. A $\frac{7}{8}$-inch hole, deep enough to hold the challenge dose, was bored into one end of the pellet. The appropriate volume of spore suspension was placed in the pellet and the hole was closed with a small cork. Each pellet was filled just before it was offered to the animal. The pellets were usually voluntarily consumed, but forcible feeding was occasionally necessary.

## EXPERIMENTAL PROCEDURES AND RESULTS

*First Immunization Experiment*—A total of 305 steers were injected subcutaneously behind the shoulder with 5 ml. of antigen preparation 690. Eleven days later, 150 of them were given a second, similar injection behind the opposite shoulder. No local or systemic reactions were noticed.

Challenge A.—Three months after immunization, 25 of the steers that had received one injection and 4 nonimmunized control steers were challenged with $1.5 \times 10^8$ spores (table 2). Twelve (48%) of the immunized steers and 3 of the 4 control steers died of anthrax. Of the 35 unimmunized control animals challenged in all experiments, 26 (74%) died of anthrax after a single challenge. Thus, it appeared probable that the single injection produced immunity that could be detected after three months, but it was evident that it did not confer satisfactory resistance against the standard challenge dose. Accordingly, two injections of antigen were used in the subsequent experiments.

Challenge B.—Six months after the first injection of antigen, 20 of the steers that had received two injections, and 9 controls, were challenged in the same manner. Inadvertently, the remainder of the vial of spore suspension that had been used in challenge A was used for challenging the first animals in this group. No deaths occurred among immunized or control animals known to have received this spore suspension. Accordingly, seven days after the initial challenge, all of the surviving animals were rechallenged with active suspension. The combined results of the two challenges are summarized (table 2). Thirteen (65%) of the 20 immunized animals died of anthrax, indicating that the two-dose immunization did not produce immunity that was effective after six months.

*Second Immunization Experiment.*—The low level of immunity obtained in the first experiment was unexpected because of the indications obtained by Schlingman *et al.*[9] that immunity persisted eight and 12 months after injection of 2, 5-ml. doses of similar preparations of antigen. Since the interval of 11 days between the two injections of antigen might have been too short to permit a true secondary response with development of satisfactory immunity, a new group of 25 steers was given 2, 5-ml. doses of antigen with an interval of one month between them. Preparation 761, concentrated twenty-fold from the culture filtrate, was used. An equal number of controls were kept with the immunized animals. At a later date, 6 normal steers were injected subcutaneously, 3 behind the shoulder and 3 in the caudal fold, with 1 ml. of living, nonencapsulated spore vaccine.[7] These animals were challenged with the final group; the interval between their immunization and challenge was three months. Animals immunized with protective antigen were challenged one month, three and a half months, and

**TABLE 2—Challenge of Immunized and Control Cattle**

| | Immunization | | | Challenge of immunized animals | | | Challenge of controls | | |
|---|---|---|---|---|---|---|---|---|---|
| Challenge experiment | Antigen preparation | No. of and interval between injections | Interval between last injection and challenge (months) | No. surviving per No. challenged | Survival time of animals that died (days) | No. infected* per No. surviving | No. surviving per No. challenged | Survival time of animals that died (days) | No. infected* per No. surviving |
| A | 690 | 1 | 3.0 | 13/25 | 4, 4, 4, 4 5, 5, 5, 5 5, 5, 6, 7 | .... | 1/4 | 4, 7, 7 | .... |
| B | 690 | 2 (11 days) | 5.7 | 7/20 | 3, 3, 4, 4 5, 5, 5, 5 5, 5, 6, 6, 9 | .... | 2/9 | 3, 4, 4, 4, 4, 6, 7 | .... |
| C | 761 | 2 (30 days) | 1.0 | 8/8 | .... | 0/8 | 2/8 | 4, 4, 4, 4, 6, 7, | 1/2 |
| D | 761 | 2 (30 days) | 3.5 | 6/8 | 4, 5, | 3/6 | 1/8 | 3, 4, 4, 4, 4, 4, 5 | 0/1 |
| E | 761 | 2 (30 days) | 7.0 | 3/8 | 5, 5, 6, 6, 7 | 2/3 | 1/6 | 3, 4, 4, 4, 6 | 0/1 |
| | Spore vaccine | 1 | 3.0 | 6/6 | | 6/6 | .... | .... | .... |

* Temperature above 102.5 F. or complement-fixation titer of 1:60 or above were considered evidence of infection.

seven months after the second injection of antigen.

Facilities and procedures were improved for these challenge experiments. Body temperatures were recorded daily, and blood samples were cultured for several days after challenge. In challenges D and E, serum samples were taken before challenge and at intervals thereafter for study of complement-fixation titers. Again, necropsies were conducted on all animals that died. The results are summarized (table 2).

Challenge C.—One month after the second injection of antigen, 8 immunized animals and 8 controls were challenged. All of the immunized animals survived, and none showed a significant rise in temperature or other signs of anthrax. Of the 8 controls, 6 died of anthrax and another was severely ill and its temperature rose to 103 F.

Challenge D.—Three and a half months after immunization, when similar groups of animals were challenged, 6 of 8 immunized animals and 2 of 8 controls survived. All survivors were rechallenged eight days after the initial challenge, and 1 additional control animal died four days after this challange. The deaths of 2 of the immunized animals suggested that the immunity of the animals had declined during the interval between challenges C and D.

Challenge E.—Seven months after the second injection of antigen, 8 immunized animals and 6 controls were challenged. The 6 steers that had received the spore vaccine three months previously were challenged at the same time. Two control animals survived this challenge, and all surviving animals were rechallenged after seven days; 1 additional control animal died. The deaths of 5 of the 8 immunized steers indicated that the immunity had declined significantly from the level attained one month after immunization. All of the animals that had received spore vaccine survived, although there was evidence of infection in all.

*General Features of the Infection.*—The earliest sign of infection in most animals was an elevation in body temperature to greater than 102.5 F. The initial elevation was noted on the first to the eighth day after challenge, and occurred on the second day in 15 per cent of the animals, on the third day in 60 per cent, and on the fourth day in 15 per cent. The highest temperature recorded was 106.9 F. and, of the 45 animals that died during the last four challenge experiments, 36 had shown temperatures of 103.0 F. or greater, and 32 of 104.0 F. or greater, on at least one day. The daily recording failed to detect temperatures greater than 102.5 F. in 4 of the animals that died, all of which were control animals that died within four days after challenge.

Marked depression, rapid pulse, glazed and staring eyes, muscular tremors, and debility were commonly observed as the infection progressed. Evidence of "colicky" pains and blood-tinged diarrhea were occasionally seen. Steers with elevated temperatures would still eat and drink and, in some cases, close observation was necessary to detect abnormal behavior.

In the terminal stages, signs of illness were pronounced, especially respiratory distress. Breathing was accelerated and labored with the mouth held open, tongue protruding, and nostrils dilated. Blood-tinged, foamy mucus would often bubble from the mouth and nostrils, and the animal became completely prostrate. Bleeding from the anus was observed occasionally, and prolapse of the rectum was noted in 1 steer.

Samples of blood, for culturing, were routinely drawn into sodium citrate solution from over half the animals in the challenge B group, and from all of the subsequent animals. Samples were obtained on the first or second day after challenge and daily thereafter until none of the survivors showed evidence of acute infection. Pour plate cultures were prepared with approximately 1 ml. of blood in nutrient agar, except that, in challenge E, Difco brain-heart infusion agar was substituted. The plates were incubated at 37 C. and examined for typical colonies of *B. anthracis* after 24 and 48 hours.

Positive cultures were obtained from most of the animals before death. During challenge E, when special efforts were made to prepare the cultures within a few minutes of the time the blood was drawn, positive cultures were obtained before death from all of the 10 animals that died. The *B. anthracis* colonies per plate ranged from one to a total too numerous to count. The longest survival after the first positive culture was 46 hours, but most animals died within 24 hours of the time of the initial positive blood culture. None that had shown a positive blood culture survived. Numerous encapsulated bacilli were seen in stained smears of the blood of all animals that died.

*Gross Pathology.*—The 19 animals that died of anthrax were examined within four hours of death. The time between challenge and death ranged from three to six days. Rigor mortis was frequently absent or incomplete, and decomposition of tissue was rapid. The blood was thick, dark red to black, and failed to clot readily. Free blood occasionally flowed from the anus and, less frequently, from the nostrils. Edematous swellings were present along the neck, flanks, or lumbar region in about one third of the steers. Enlargement of the prescapular and prefemoral lymph nodes could be detected in 4 animals prior to incision.

At necropsy, the animals bled freely from the cutaneous blood vessels. Subcutaneous hemorrhages were common and varied in size. Large, clearly demarcated areas of hemorrhagic edema with a yellowish, gelatinous infiltration were present over the lumbar region near the midline. Hemolymph nodes were enlarged and numerous.

The organs are described in the order of decreasing frequency of gross lesions:

Spleen.—The spleen presented the most characteristic and constant gross pathological changes. It was always enlarged, varying from 22 to 31 inches in length, 8 to 10 inches in width, and 2 to 3 inches in thickness at the middle third. A few spleens were only slightly enlarged in length and width, although all were markedly thickened.

The spleens were soft and friable. Several showed bright red lines of subcapsular hemorrhages which radiated diagonally across the parietal surface. A few spleens were diffusely hemorrhagic. Some showed large subcapsular ecchymoses, often approaching suffusions, while others showed only a few small ecchymoses. The cut surface bulged and the contents consisted of a semifluid homogeneous mass of degenerated splenic pulp. Trabeculae and Malpighian corpuscles could not be detected. The splenic pulp varied from dark red to black.

Small Intestine.—An edematous and hemorrhagic area in the small intestine, 4 to 6 inches long and located approximately 16 feet from the pylorus, was seen in 18 of 19 steers. Profuse hemorrhage and edema into this section caused a clear line of demarcation from the contiguous bowel. The hemorrhage extended from the serosa through the mucosa, and the lumen of the bowel contained free blood and degenerated mucosa. In the adjacent mesentery, the blood vessels were markedly engorged and occasionally ruptured, and the lymph nodes were about twice their normal size, with massive hemorrhage in both cortex and medulla. The mucosa of the remainder of the small intestine showed scattered petechiae and ecchymoses, and the contents of the lumen were often mixed with free blood.

Lymph Nodes.—One or more lymph nodes in each steer showed gross pathological changes. A typical case might show hemorrhage and edema in the prescapular nodes with no visible involvement of the prefemorals. The prescapular, prefemoral, bronchial, mediastinal, and mesenteric nodes were the most frequently involved. The dorsal mediastinal nodes, situated on either side of the thoracic aorta, were edematous and hemorrhagic in every steer. The gross changes in the nodes varied from a slight to moderate edema with petechiae, to diffuse hemorrhage in both cortex and medulla. Perilymph tissue was often infiltrated with hemorrhage and edema, so that the nodes appeared abnormally large.

Lungs, Trachea, and Epiglottis.—Congestion of the lungs was apparent in 8 of the animals, with 2 showing localized areas of alveolar emphysema

surrounded by small hemorrhages. The tracheas of several animals contained a bloody froth and 2 showed a few scattered petechiae. No gross lesions were noticed in the epiglottis.

Kidneys and Urinary Bladder.—Congestion of the kidneys was observed in 7 of the 19 animals, with marked pathological changes in only 2 steers. The kidneys of both these steers were approximately one and a half times their normal size, and the cut surfaces showed diffuse hemorrhage in the cortex and medulla. There was massive hemorrhage and gelatinous infiltration into the perirenal fat. The urinary bladders appeared normal, except in 3 animals in which the mucosae showed congestion with petechiae in some areas.

Abomasum.—The mucosa of the abomasum was congested in 6 of the 19 animals.

Cecum, Colon, and Rectum.—Occasional linear and ecchymotic musocal hemorrhages and evidence of bleeding into the lumen of the organs were found.

Rumen, Reticulum, Omasum.—These organs appeared normal except for occasional flamelike hemorrhages on the serosa of the rumen.

Heart and Pericardium.—The heart was not involved in every steer but, when lesions were present, they were extensive. Hemorrhages sometimes extended from the epicardium through the myocardium to the endocardium. Massive endocardial hemorrhages covering both ventricles were fairly common. Scattered petechiae and ecchymoses were common in the epicardium and endocardium. Petechiae were occasionally found in the pericardium.

Liver and Gallbladder.—The liver was occasionally congested, dark red, and friable. Petechiae were observed in the gallbladders of 3 of the 19 animals.

Brain and Membranes.—The brains of 2 steers were examined. Hemorrhages were observed in the gyri of the left frontal lobe in the subarachnoid area. Vessels and branches of the circle of Willis were very congested. A serosanguineous fluid was evident in an area of the fourth ventricle and in the lateral ventricles. Petechiae were scattered throughout the brain, but were mainly concentrated in the thalamic area. The dura mater and pia mater appeared normal.

Endocrine Glands.—The only gross lesions observed in the endocrine glands were in the adrenals of 2 animals. Upon cross section, these glands exhibited congestion and hemorrhages only in the cortical portion.

Great Omentum.—Scattered ecchymoses were occasionally observed.

Tongue, Tonsils, Esophagus.—These appeared normal in every steer examined.

*Serological Studies.*—To obtain evidence of possible minimal or inapparent infection among animals that survived challenge, complement-fixation titrations were carried out on serum samples obtained before, and at intervals after, challenge. Previous studies had shown that a marked rise in titer occurred in infected survivors of several species.[5] Fixation reactions were carried out in the cold with guinea pig complement and culture filtrate that contained protective antigen. The methods are described in detail by Jones and McGann.[6] Results of the complement-fixation titrations and summaries of the body temperature records of the respective animals are given (table 3). There was a general correlation between the occurrence of a febrile reaction and the development of complement-fixation titers. Titers without febrile

**TABLE 3—Body Temperatures and Complement-Fixation Titers of Surviving Steers**

| Animal and challenge experiment | Immunization | Days elevated temperature | Highest temperature recorded (F.) | Complement-fixation titer 18 to 19 days after challenge * |
|---|---|---|---|---|
| 22–D | Alum-precipitated antigen | 0 | 101.6 | 1:100 |
| 28–D | Alum-precipitated antigen | 0 | 101.8 | — |
| 33–D | Alum-precipitated antigen | 5 | 105.4 | 1:240 |
| 35–D | Alum-precipitated antigen | 0 | 101.5 | — |
| 42–D | Alum-precipitated antigen | 1 | 103.4 | 1:800 |
| 40–E | Alum-precipitated antigen | 0 | 101.8 | 1:600 |
| 45–E | Alum-precipitated antigen | 4 | 105.6 | 1:640 |
| 70–E | Spore vaccine | 1 | 104.0 | 1:600 |
| 71–E | Spore vaccine | 2 | 104.8 | 1:600 |
| 72–E | Spore vaccine | 0 | 101.8 | 1:120 |
| 73–E | Spore vaccine | 2 | 104.6 | 1:120 |
| 74–E | Spore vaccine | 2 | 103.0 | 1:120 |
| 75–E | Spore vaccine | 0 | 102.4 | 1:120 |
| 2191–D | None | 0 | 102.4 | — |
| 2116–E | None | 4 | 105.8 | — |

* — indicates no fixation at 1:10 dilution. Serum samples obtained before challenge gave no fixation.

response were developed by 3 animals, presumably because the infection was minimal. No elevation in titer occurred in 1 normal animal that survived following an elevation of temperature for four days. The 6 animals that had received spore vaccine all showed marked elevations of titer following challenge; evidently all were infected, although febrile responses were mild or absent.

## DISCUSSION

The experiments confirm the immunizing activity of the alum-precipitated anthrax antigen in cattle, and indicate that the immunity declined significantly from the level reached one month after the second injection of antigen. The decline was more rapid than was expected on the basis of the results of Schlingman *et al.*[9] with smaller numbers of animals. Explanations for this possible difference include variations in the animals, differences in the environmental conditions, or differences between the preparations of antigen. There would appear to be no way of evaluating the relative importance of these factors.

A single injection of antigen evidently produced a certain degree of immunity, although it now appears that the interval of three months between immunization and challenge was too great to permit a clear demonstration of the effect. Two injections of antigen, with an interval of one month between them, seemed to produce more effective immunity but, since a different preparation of antigen was used in the second immunization experiment, the conclusion is somewhat tentative. It would appear that barely detectable immunity persisted seven months after immunization.

A single injection of Sterne-type spore vaccine produced effective immunity three months after immunization. It is of interest that, although all of the animals survived the challenge, there was serological evidence that all became infected, and 4 of the 6 animals showed brief elevations in temperature. Additional experiments to determine the duration of the immunity following administration of spore vaccine would be of interest.

Schlingman *et al.*[9] pointed out that there is no basis for relating the severity of the artificial challenge procedure to the severity of the natural exposure under field conditions. It is possible that the challenge procedure was much more severe than natural exposure, and that levels of immunity inadequate to withstand artificial challenge would prove protective under natural conditions of exposure. The primary site of infection, as found at necropsy, is a region of small intestine. The infection spreads via the lymphatics to the general circulation. It is generally believed that natural anthrax in cattle is usually associated with ingestion of contaminated forage.[11] Presumably, the artificial challenge procedure produces an infection not unlike the natural disease.

## SUMMARY

Two subcutaneous injections of alum-precipitated antigen produced immunity to anthrax in cattle. The resistance was effective one month after immunization, had declined somewhat after three and a half months, and was barely detectable after seven months. One challenge dose of $1.5 \times 10^8$ spores, given by mouth, was fatal to 74 per cent of the control animals. In animals that died of infection, a characteristic lesion was consistently found in the small intestine and adjacent mesenteric lymph nodes. This lesion appeared to be the portal of entry for the generalized infection. A single injection of alum-precipitated antigen was less effective than two injections. The effectiveness of a non-encapsulated living spore vaccine was also tested in a single experiment. All of 6 animals survived challenge three months after immunization, but all showed evidence of infection. Characteristics of the disease in normal and partially immune animals are discussed, and the results of necropsies are presented.

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# Distribution of Micrococci and Other Bacteria in Milk Samples from a Single Dairy Herd After Twelve Years of Mastitis Control

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A STATE hospital with a dairy herd of 250 Holstein-Friesian cows has been a major cooperator in our studies on mastitis since 1943. During the entire period, all basic management practices have been well above average in excellence.

*Streptococcus agalactiae* was eliminated from the herd between 1943 and 1945. Thereafter, the program was directed toward the control of udder infections due to the toxin-producing micrococci.[13, 18] The cows shedding micrococci which produce *alpha-beta* or *beta* toxins were detected by streaking incubated milk on the surfaces of cow blood agar plates. In addition to strict segregation of shedder cows, a variety of therapeutic agents were infused into infected glands both when lactating and when dry.[12, 15, 17]

Coliform mastitis appeared in the herd in 1947 and became a serious problem.[14] A successful method for treatment was developed, using massive doses of dihydrostreptomycin.[16] An unsuccessful attempt was made, between 1948 and 1952, to increase the resistance of the herd to coliform organisms through the use of autogenous vaccines.[11]

Since 1952, mammary quarters shedding toxin-producing micrococci have been less vigorously treated during lactation, but treatment of all known infected quarters was continued during the dry period. It was conjectured that a coccic flora present in the mammary gland during lactation may have some value in precluding the establishment of a coliform population in the udder.[20]

This paper is concerned with the distribution of bacterial types in milk samples obtained from the herd during the two-year period from 1955 to 1956, the thirteenth and fourteenth years of mastitis control.

## METHODS

*At the Dairy.*—The cows were milked by machine and stripped by hand, using a sequence of milking designed to reduce the spread of mammary gland infections. Prior to milking, the udders were cleaned by water under pressure and permitted to dry. Before the milking machine was applied, the teats were massaged with a clean cloth containing a chlorine disinfectant (200 p.p.m.). The teats were again disinfected after hand stripping by dipping them into the chlorine solution.

Before drawing milk samples for bacteriological study, an employee of the dairy, trained in the proper technique, applied a strong sodium hypochlorite solution (1 oz. 70% available chlorine per 1 gal. of water) to the teats, using one or two clean washcloths for each udder. He wore rubber gloves to protect his hands from irritation by the strong chlorine solution. About 150 milk samples were drawn from the herd each week for bacteriological study.

In sampling, the first stream of milk was discarded and then about 15 ml. was drawn into a sterile screw-cap vial containing 1.0 ml. of a 1:300 bromeresol purple solution. Composite samples were drawn from individual cows when they were being sampled routinely, but individual quarter samples were taken from fresh cows within the week of calving, from "drying-off" cows, and from all cows when verification of infection was desired, or following intramammary therapy. When dry cows were sampled, the bromeresol purple was eliminated from the vial. The samples were incubated at 37 C. for 18 to 20 hours at the dairy and then refrigerated at 7 C. until transported (60 miles) to the laboratory.

Samples for the Whiteside test,[9] which was conducted in parallel with the bacteriological studies during part of the investigation, were drawn at the same time and kept under refrigeration.

*In the Laboratory.*—The Hotis test reactions were recorded on arrival of the samples at the laboratory. Then a loopful of thoroughly mixed sample was streaked on half of the surface of a tryptose, cow blood agar plate.[18] After incubation at 37 C. for 18 to 20 hours, the plates were carefully examined for characteristic growth of the various microorganisms common to the herd, and a tentative identification was made.

Preliminary classification of Micrococcus colonies was made as follows: M = no change in the agar surrounding the colony; M′ = a narrow zone of hemolysis, usually less than 1 mm. beyond the edge of the colony; AB = presence of *alpha-beta* toxins; and B = presence of *beta* toxin only.

Pure cultures of micrococci were established on phenol-red, mannitol agar slants containing 7.5 per

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cent salt.[3] Ability to coagulate rabbit plasma[4] was determined with young subcultures from these original slants. The code letters M and M′ assigned to a culture on the basis of the original growth on the surface of the cow blood agar plate were modified for coagulase-positive strains by addition of a plus (+) sign. Micrococcus cultures designated as M+, M′+, B, or AB were regarded as pathogenic micrococci and were called *Micrococcus pyogenes*. The M and M′ cultures were coagulase negative and considered to be nonpathogenic micrococci.

Cultures of other microorganisms were established in serum broth for use in pure culture studies when desired for specific identification. Coliform bacteria were differentiated by the "IMVIC" method[5] and their ability to ferment lactose, sucrose, and glycerol. *Pseudomonas aeruginosa* was identified by colony characteristics, typical odor, and production of the chloroform-soluble pigment, pyocyanin. Streptococci, Proteus, and *Corynebacterium pyogenes* were identified on the basis of characteristic growth patterns on the surface of cow blood agar, and by action on litmus milk and on selected carbohydrates when deemed essential. Udder diphtheroids and soil organisms were grouped as "other bacteria" and identified by colony morphology.

The Whiteside test was conducted on a glass plate using 5 drops of cold milk and 1 drop of 4 per cent sodium hydroxide. The reactions were scored negative through 4+, but for the purpose of this paper all 1+ reactions or greater are grouped as Whiteside-positive milk samples.

## Results

Composite and individual quarter milk samples, totaling 17,927, were obtained for bacteriological culture from this single dairy herd in the two-year period covered by this report. The data relative to number of cows and distribution of the samples by lactation age are shown (table 1). Since, in a single calendar year, a cow could complete one lactation and begin the next, lactation pairs were used; *i.e.*, 1-2, 2-3, 3-4, 4-5, and 5 and over. Each cow, moving from one lactation pair to the next during the two-year period, was treated as two separate animals. There was a gradual increase in the average number of samples collected per cow with advancing lactation age. This resulted from the practice of drawing individual quarter samples from cows previously demonstrated to be shedding bacteria that were potentially pathogenic, as well as from the increased frequency of sampling such cows. The average number of samples increased from 21 per cow in the first lactation pair, to 47 per cow in the fifth lactation pair.

**TABLE 1—Distribution of Cows and Milk Samples Among the Five Lactation Pairs**

| Lactation pair | No. of cows | No. of milk samples | | Total milk samples in each lactation | Average No. samples per cow |
|---|---|---|---|---|---|
| | | Composite | Quarter | | |
| 1-2 | 249 | 1,004 | 4,331 | 5,335 | 21.4 |
| 2-3 | 103 | 651 | 2,806 | 3,457 | 33.6 |
| 3-4 | 113 | 602 | 3,209 | 3,811 | 33.7 |
| 4-5 | 54 | 220 | 2,043 | 2,263 | 41.9 |
| 5 and over | 65 | 301 | 2,760 | 3,061 | 47.0 |
| Total | 584 | 2,778 | 15,149 | 17,927 | 30.7 |

A total of 12,784 bacterial cultures appeared on the blood agar streaked with the 17,927 milk samples. The bacterial types and their distribution among the lactation age groups are shown (table 2). The distribution of the various microorganisms on a percentage basis varied only slightly between the lactation age groups. The mean distribution for all lactation pairs was: micrococci (70.8%), coliform organisms (11.2%), streptococci (8.5%), Proteus (1.0%), *Ps. aeruginosa* (0.07%, *C. pyogenes* (0.2%), and all other bacteria (8.2%).

Within the genus Micrococcus, lactation age significantly affected the ratio of pathogenic to nonpathogenic types among the bacteria isolated. In general, the coagulase-positive strains increased as lactation age advanced (table 3 and graph 1). The M+ type constituted 6.0 per cent of the micrococcic isolates from the first lactation pair. The incidence then fell, but increased to 13.0 per cent in the fifth lactation pair. The M′+ type constituted 9.6 per cent in the first lactation pair and increased gradually to 16.0 per cent. Micrococci which produced *beta* or *alpha-beta* toxins increased from 9.7 per cent from the first lactation pair to 29.6 per cent in the fourth pair, followed by a drop to 17.9 per cent in the fifth pair. All morphological types of coagulase-positive micrococci were combined (graph 1); *i.e.*, M+, M′+, B, and AB. Pathogenic micrococci were represented among the micrococci isolated from each of the five lactation pairs, respectively, e.g. 25.4 per cent, 27.1 per cent, 33.9 per cent, 46.3 per cent, and 46.8 per cent.

Whiteside test results are compared with bacterial flora (table 4). Infrequently, a milk sample supported growth of two distinct types of bacteria. In such instances, the organisms were listed separately and correlated with the Whiteside reaction of

**TABLE 2—The Number and Percentage Distribution of Major Groups of Microorganisms Among the Five Lactation Pairs**

| Types of microorganisms | Lactation pairs | | | | | Total from all lactation pairs |
|---|---|---|---|---|---|---|
| | 1-2 | 2-3 | 3-4 | 4-5 | 5 and over | |
| Micrococci | 2,372 | 1,849 | 1,975 | 1,171 | 1,685 | 9,052 |
| | 72.0% | 70.1% | 69.5% | 71.6% | 71.0% | 70.8% |
| Coliform | 375 | 322 | 328 | 163 | 238 | 1,426 |
| | 11.4% | 12.2% | 11.5% | 10.0% | 10.0% | 11.2% |
| Streptococci | 221 | 243 | 252 | 128 | 244 | 1,088 |
| | 6.7% | 9.2% | 8.9% | 7.8% | 10.3% | 8.5% |
| Proteus | 8 | 18 | 46 | 2 | 57 | 131 |
| | 0.2% | 0.7% | 1.6% | 0.1% | 2.4% | 1.0% |
| *Pseudomonas aeruginosa* | 3 | 3 | 1 | .... | 2 | 9 |
| | 0.09% | 0.1% | 0.04% | .... | 0.08% | 0.07% |
| *Corynebacterium pyogenes* | 9 | 13 | 5 | 1 | 1 | 29 |
| | 0.3% | 0.5% | 0.2% | 0.06% | 0.04% | 0.2% |
| Other bacteria | 307 | 189 | 235 | 170 | 148 | 1,049 |
| | 9.3% | 7.2% | 8.3% | 10.4% | 6.2% | 8.2% |
| Total | 3,295 | 2,637 | 2,842 | 1,635 | 2,375 | 12,784 |

the milk in which their presence was detected. Among 2,616 samples of milk containing no significant bacteria, 14.5 per cent gave positive Whiteside reactions. Included in this group are samples from udders that had been treated for infections, with subsequent disappearance of the infective agent but with persistence of a high leukocyte count in the milk. Whiteside-positive reactions equal to between 19.5 per cent and 23.0 per cent occurred among milk samples containing the coagulase-negative micrococci and nonagalactiae streptococci. When coliform organisms were isolated, such milk samples exhibited a 41 per cent level of Whiteside-positive reactions. Milk samples containing the pathogens *M. pyogenes* and *Str. agalactiae* were positive to the Whiteside test at the 50 per cent level.

## DISCUSSION

The distribution of the various major groups of microorganisms common to this herd was relatively uniform among the 5 lactation pairs established on the basis of advancing lactation age. Micrococci were the most prevalent (70.8%).

Ward,[22] in 1900, cultured 19 apparently normal udders of cows slaughtered as reactors to the tuberculin test. He found bacteria, nearly all of which were micrococci, throughout the lactiferous ducts. Harding and Wilson,[7] in 1913, examined 1,230 stripping samples taken from 78 cows in which the bacteria were practically all Gram-positive, and 75 per cent of these were micrococci. Murphy[8] cultured 15,000 milk samples from 195 cows and found 77 per cent of all bacteria to be micrococci, fluctuating within the narrow range of 72 to 83 per cent when separated into five lactation groups.

Evans,[6] in 1916, published a report on the pathogenic nature of micrococci found in 192 milk samples coming from udders regarded as normal and from five dairy herds. Streptococci were found in 15 per cent of the samples and micrococci in 59 per cent. She concluded that about 50 per cent of Micrococcus cultures, derived from the so-called "normal" milk, showed characteristics of the pathogenic type.

The genus Micrococcus consists of many separate species which fall into two groups with respect to ability to coagulate rabbit plasma. Cruickshank[3] called attention to the production of coagulase by pathogenic micrococci. This enzyme is produced by all pathogenic strains, irrespective of toxin characteristics, and is absent in cultures of saprophytic micrococci. Elek and Levy[5] state that coagulase-positive micrococci may produce one or more of three distinct hemolysins (toxins), *i.e., alpha, beta,* and *delta* hemolysins. On the other hand, coagulase-negative micrococci do not produce true toxins, but many strains may show uninhibited hemolysis (designated by the letter E).

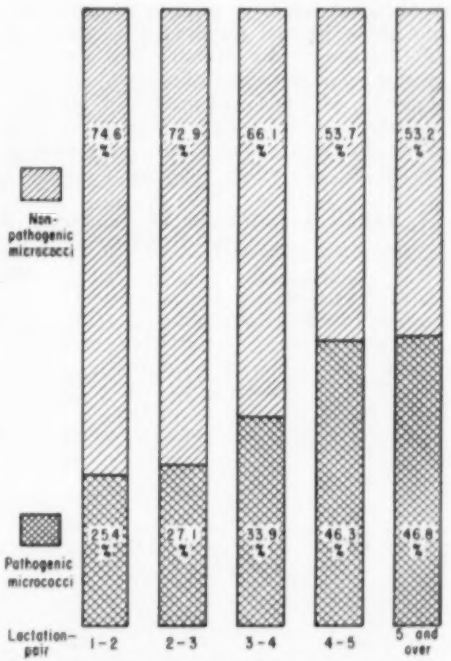
The cow blood agar used in the current study demonstrates *beta* toxin as a darkened zone of erythrocytes extending from 3 to 10 mm. beyond the colony edge. The *alpha* toxin appears as a narrow zone of true hemolysis extending not more than 1 to 2 mm. beyond the edge of the colony. When *alpha* toxin is present without accompanying *beta* toxin, it is impossible to distinguish it by direct inspection of the agar plate from the type of hemolysis designated as E by Elek and Levy. *Delta* toxin can not be observed directly on cow blood agar.

Early in the program of control of the toxigenic micrococci in the herd under consideration, an analysis was made of colony characteristics of coagulase-positive strains regularly isolated from the milk samples.[13] It was shown that 90 per cent of coagulase-positive micrococci produced *beta* or *alpha-beta* toxins. In the eleventh and twelfth years after control of *Str. agalactiae* infection was achieved, less than half (47%) of the coagulase-positive micrococcic cultures could be detected by direct inspection of the blood agar plate for presence of the *beta* toxin zone. Slanetz and Bartley,[21] as well as Schalm and Woods,[18] have indicated that, for routine purposes, the sheep or cow blood agar plate may be used for direct selection of pathogenic micrococci on the basis of toxin manifestation. The former authors reported that cultures which produced hemolytic zones of less than 1 mm. were nearly 100 per cent coagulase-negative, and that all of 232 strains of nonhemolytic micrococci were coagulase-negative. Early studies in this herd revealed that, of 170 nonhemolytic micrococcic cultures, only 4 per cent coagulated rabbit plasma, but now, 18 per cent of the 2,763 nonhemolytic cultures must be regarded as pathogenic on the basis of positive coagulase reaction. Thus it appears that as spread of the toxigenic micrococci was curtailed, the nonhemolytic coagulase-positive micrococci increased, and the cow blood agar plate became less reliable as a direct means for detection of the pathogenic micrococci in this herd.

Although micrococci as a group (about 70% of all organisms) did not vary significantly with lactation age, the percentage of pathogenic strains, on the basis of coagulase reaction, increased as the cows advanced in lactation age. While about 46 per cent of the micrococci isolated from the older cows must be considered as pathogenic, this was considerably lower than would be encountered in herds in which control measures directed against the toxigenic micrococci were not employed.[19] Thus, despite many years of intensive effort, the best that could be accomplished was a measurable reduction in the occurrence of such microorganisms.

The coliform organisms showed a uniform distribution (10 to 12%) among the various lactation age pairs. Some of these, no doubt, were contaminants which entered the milk at the time of sampling. When coliform mastitis was at its peak in the herd, 96 quarters among 270 cows were classified as infected.[16]

*Streptococcus agalactiae* was eradicated in 1945, and the herd remained free from this pathogen until November, 1954, when it reappeared. As the source of the reintroduced *Str. agalactiae*, circumstantial evidence pointed to a person who milked the fresh and hospitalized cows between the hours of 1:00 to 4:00 a.m. and who worked in a creamery between 5:00 p.m. and mid-




Graph 1—Ratio of nonpathogenic to pathogenic micrococci in udder flora as influenced by lactation age.

night. Thus, within an hour after leaving the creamery, which received milk from many different farms, he was manipulating the udders of cows in the hospital barn. He may have brought *Str. agalactiae* to the dairy on his hands or clothing. The first occurrence of *Str. agalactiae* was in one quarter of a lame cow that had been in the hospital barn for several months. Later *Str. agalactiae* was found in 3 fresh cows that recently had been in the hospital barn. Despite early detection, 29 cows became infected (total 36 cultures isolated) before this pathogen was again eliminated (table 4).

In all, 1,088 cultures of streptococci were observed. *Streptococcus dysgalactiae* and *Streptococcus uberis* were identified from time to time; but, in general, the Streptococcus cultures that were tested by carbohydrate fermentation could not be named specifically and, for the most part, were considered to be saprophytic in character. The remaining types of bacteria listed (table 3) did not occur with sufficient frequency to merit discussion.

The Whiteside test is a presumptive method for detection of milk samples having above normal numbers of leukocytes. In this herd, milk samples that were regarded as negative for significant bacteria showed a 14.5 per cent level of Whiteside-positive reaction. Some probably were from quarters which were approaching drying-off or which had been recently treated for specific infections. Milk from drying-off glands may give a positive Whiteside reaction due to increased cell content. Following udder infusions administered to fully lactating udders, milk may remain positive to the Whiteside test for some time after disappearance of the infection. The level of 14.5 per cent Whiteside positive may be taken as a base to which Whiteside reactions in milk containing significant bacteria may be compared.

The Whiteside test showed the highest correlation of positive reactions (50%) with milk containing the coagulase-positive micrococci and *Str. agalactiae*. It is interesting that there was no significant difference in the percentage of Whiteside-positive reactions between the different types of coagulase-positive micrococci. Thus, it would appear that the irritational qualities of coagulase-positive micrococci in the mammary gland are not entirely dependent on the presence of *beta* or *alpha-beta* toxins. As stated above, a third toxin (designated *delta*) is produced by some micrococci. The methods used in this study did not permit the demonstration of *delta* toxin.

The coagulase-negative micrococci were associated with Whiteside-positive reactions of about 20 per cent. This was 5 per cent above the base level of 14.5 per cent in milk samples not containing significant bacteria, and may indicate that coagulase-negative micrococci have properties that could lead to tissue irritation. Deubler and Cole,[4] in

**TABLE 3—Distribution of the Various Morphological Types of Coagulase-Positive and Coagulase-Negative Micrococci Among the Lactation Pairs**

| Morphological types of Micrococcus * | Lactation pairs | | | | | |
|---|---|---|---|---|---|---|
| | 1-2 | 2-3 | 3-4 | 4-5 | 5 and over | Totals from all lactation pairs |
| M | 760 | 472 | 462 | 248 | 323 | 2,265 |
| | 32.0% | 25.5% | 23.4% | 21.2% | 19.1% | 25.0% |
| M′ | 1,010 | 874 | 844 | 381 | 573 | 3,682 |
| | 42.6% | 47.3% | 42.7% | 32.5% | 34.0% | 40.7% |
| M+ | 143 | 43 | 74 | 19 | 219 | 498 |
| | 6.0% | 2.3% | 3.7% | 1.6% | 13.0% | 5.5% |
| M′+ | 228 | 206 | 272 | 176 | 269 | 1,151 |
| | 9.6% | 11.1% | 13.8% | 15.0% | 16.0% | 12.7% |
| AB/B | 231 | 254 | 323 | 347 | 301 | 1,456 |
| | 9.7% | 13.7% | 16.4% | 29.6% | 17.9% | 16.1% |
| Totals | 2,372 | 1,849 | 1,975 | 1,171 | 1,685 | 9,052 |

* M = Nonhemolytic, coagulase-negative; M′ = weakly hemolytic, coagulase-negative; M+ = nonhemolytic, coagulase-positive; M′+ = weakly hemolytic, coagulase-positive; AB/B = coagulase-positive, *alpha-beta* or *beta* toxin only.

**TABLE 4—Correlation of Whiteside Test Results with Bacterial Flora**

| Whiteside reaction | No significant bacteria | Micrococci [a] Coagulase-negative M | M′ | Coagulase-positive M+ | M′+ | AB/B | *Str. agalactiae* | Nonagalactiae streptococci | Coliforms | Totals |
|---|---|---|---|---|---|---|---|---|---|---|
| Whiteside-negative | 85.5% | 79.6% | 80.5% | 47.0% | 47.7% | 49.5% | 47.0% | 77.0% | 59.0% | 75.0% |
| Whiteside-positive | 14.5% | 20.4% | 19.5% | 53.0% | 52.3% | 50.5% | 53.0% | 23.0% | 41.0% | 25.0% |
| Total samples | 2,616 | 652 | 1,244 | 204 | 426 | 398 | 36 | 257 | 413 | 6,246 |

[a] M = Nonhemolytic; M′ = weakly hemolytic; AB/B = *alpha-beta* or *beta* toxin.

studying a single herd of 45 cows over a period of one year, made the observation that the majority of micrococci isolated from clinical cases of mastitis in that herd showed neither hemolysis nor coagulase activity.

Milk samples containing the nonagalactiae streptococci showed levels of Whiteside-positive reaction similar to those found in samples containing the saprophytic micrococci, while the samples containing *Str. agalactiae* had much higher percentages of Whiteside-positive reaction.

Coliform bacteria, which in the past had been associated with serious acute mastitis in the herd, were intermediate between the saprophytic and pathogenic micrococci in the level of Whiteside-positive response of milk samples in which they were contained. No doubt, some instances of occurrence of coliform bacteria in milk samples were the result of contamination at the time of sampling. If it were possible to delete the contaminated samples from the data, the Whiteside-positive reactions of milks containing coliform bacteria would exceed the 41 per cent reported (table 4).

## SUMMARY

1) In a *Streptococcus agalactiae*-free herd, the micrococci comprised 70 per cent of the types of bacteria isolated from foremilk, regardless of the lactation ages of the cows.

2) The coagulase-positive, pathogenic micrococci showed an increase in occurrence as lactation age advanced. This was accompanied by a decrease in the coagulase-negative, saprophytic micrococci, so that the sum of these two groups of micrococci remained the same.

3) Pathogenic micrococci that produce *alpha-beta* or *beta* toxins can be detected by growth patterns on cow blood agar. However, coagulase-positive micrococci exist that produce no visible change in the agar, or produce only a slight hemolysis. Such strains of pathogenic micrococci can not be distinguished, with any degree of accuracy, from the coagulase-negative, saprophytic micrococci by growth patterns on cow blood agar. For this reason, the coagulase test must be applied to all strains of micrococci not exhibiting *beta* toxin when the intent is to detect all pathogenic micrococci present in a series of milk samples.

4) *Streptococcus agalactiae* was eradicated with comparative ease within a two-year period. On the other hand, the number of toxigenic *Micrococcus pyogenes*, while reduced, was not completely eliminated, even after 12 years of application of vigorous control measures specifically directed against it.

5) *Streptococcus agalactiae* reappeared after ten years, and spread to more than 10 per cent of the herd before being eliminated again. Circumstantial evidence pointed to reintroduction of the pathogen on the hands or clothing of a milker who was permitted to hold a second job in a creamery receiving milk from many dairy farms.

6) The percentages of positive Whiteside reactions in milk and the associated microorganisms were: no significant bacteria (14.5%), coagulase-negative micrococci (20.0%), nonagalactiae streptococci (23.0%), coliform organisms (41.0%), coagulase-positive micrococci (52.0%), *Str. agalactiae* (53.0%).

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# Direct Complement Fixation for Detection of Foot-and-Mouth Disease Antibody in Serums from Experimentally Infected Cattle

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ALTHOUGH INDIRECT complement-fixation, neutralization, and protection tests,[3, 16] are reliable and useful for the detection of foot-and-mouth disease virus (FMDV) antibodies in the serums of convalescent or vaccinated cattle, attempts to employ the direct complement-fixation test for this purpose have not met with similar success.[4, 16] The present report describes a method for direct complement (c′) fixation with serums from cattle which have been experimentally infected with FMDV. The appearance and disappearance of complement-fixing antibodies in individual animals have been studied. Complement-fixing antibodies have been detected in high titer in all the cattle infected with the three common types of FMDV. A subsequent report will be concerned with the demonstration of direct c′ fixation with serums from vaccinated cattle.

## MATERIALS AND METHODS

*Antiserums.*—Blood was taken from healthy cows for normal serum and the cows were then infected with FMDV by intradermolingual inoculation of approximately $10^7$ bovine infective doses. The animals which were inoculated with type $A_5$ and type $C_1$ viruses, had been imported into Denmark from Sweden. Danish cows were used for the type $O_1$ experiment. The cattle were kept in isolation stalls for the entire experimental period. Blood samples were taken from the jugular vein twice a week for the first month following infection, and once a week thereafter for another month unless otherwise indicated. The serums were separated from the clotted blood, heated for one half hour at 56 C., and then stored at −40 C. until used. They were usually assayed one to three days after collection. Immediate assay is not necessary, however, since the antibody appears to be stable under these conditions of storage for at least four months, the maximum period of storage on which results are available.

The lowest dilution of cattle serum employed in the c′-fixation analyses was 1:15. This was necessary because lower dilutions sometimes gave strong conglutination.

*Antigens.*—The antigen types employed were stock strains of $O_1$, $C_1$, and $A_5$ viruses which were on hand at the Lindholm Island Laboratory. These have been passaged through cattle for many years and are the strains regularly employed for the production of vaccine in Denmark.

Vesicular fluid from tongue lesions of 20 to 30 cows, for each type of virus, was pooled and used as antigen. The cattle were inoculated with virus on the afternoon previous to collection of the fluid. At first, the fluid was preserved as a 50 per cent solution with 25 per cent phosphate buffer (pH 7.5) and 25 per cent glycerol. Later, lots of fluids were not diluted with this buffer and were satisfactory for use in the c′ fixation test at a dilution of 1:60. After collection, the vesicular fluid was centrifuged at 1,000 x g for one half hour and stored at −40 C. without further treatment.

In several experiments, a 33 per cent (w/v) suspension of infected tongue epithelium was employed as antigen. The finely sliced tongue material was suspended in veronal buffer and ground in a mortar with the aid of sterile quartz granules. The resulting suspension was centrifuged at 1,000 x g for one half hour, and the opalescent supernatant fluid used as antigen.

For tissue-culture antigen, a 14-liter culture was prepared at Lindholm following the method of Frenkel.[5] After 22 hours, a portion which contained both tongue material and fluid was removed. The tongue epithelium was ground in a mortar with quartz granules, after which the mixture was centrifuged and filtered through a Seitz filter. The filtrate represented the undiluted tissue-culture virus antigen. Its i.d.$_{50}$ in unweaned mice was between $10^{-6}$ and $10^{-7}$.

*Diluent.*—Veronal buffer containing 0.0005 M magnesium ions and 0.00015 M calcium ions was used as diluent. Since the components of the complement-fixation test were used as dilute solutions, 0.1 per cent gelatin was incorporated in the buffer to minimize loss of proteins or virus.

*Erythrocytes.*—Sheep blood, drawn aseptically into an equal volume of sterile, modified Alsever's solution,[5] was stored at 2 to 5 C. The cells were permitted to stabilize for four days before use.

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The desired quantity of cells, taken from the Alsever solution using sterile technique, was centrifuged at 1,000 x g for ten minutes and washed three times with ten volumes of isotonic veronal buffer. A 5 per cent suspension was made by adding 1 ml. of the packed, washed cells to 19 ml. of veronal buffer. One milliliter of this suspension was then lysed in 14 ml. of 0.1 per cent $Na_2CO_3$. The optical density of the resulting clear red solution was determined with the Beckman DU spectrophotometer at a wave length of 541 $\mu$ in 10-mm. cuvettes, with 0.1 per cent $Na_2CO_3$ as reference blank. An optical density of 0.68 under these conditions corresponds to $10^8$ cells per milliliter in the original 5 per cent suspension. When the optical density reading obtained is too high, veronal buffer is added to the preliminary 5 per cent cell suspension. To obtain a correct reading, veronal buffer is added according to the following formula:

$OD_1$ = optical density of preliminary 5 per cent cell suspension; $V_1$ = volume of preliminary 5 per cent cell suspension after removal of 1 ml.; and $V_2$ = final volume of adjusted cell suspension.

$$\frac{OD_1}{0.680} \times V_1 = V_2$$

To the final cell suspension containing $10^8$ cells per milliliter (OD = 0.680) is added an equal volume of the appropriate amboceptor dilution resulting in $0.5 \times 10^8$ cells per milliliter. In the c′-fixation analyses, 0.2 ml. of sensitized cells ($10^8$ cells) is employed.

*Complement* (c′).—Thirty healthy adult guinea pigs were exsanguinated by cardiac puncture. The serum was removed from the pooled, clotted blood, centrifuged in the cold (0 to 2 C.), and stored at —40 C. in 2 ml. amounts in rubber-stoppered test tubes. All these procedures were carried out in one day, and the c′ was always kept cold. Under these conditions of storage, the complement titer is constant over a long period. The c′ titer was determined as described by Osler, Strauss, and Mayer.[12]

*Amboceptor.*—Rabbit anti-sheep erythrocyte antibody was prepared by administering 12 intravenous inoculations, three per week, of thoroughly washed and boiled (20 min.) sheep cell stroma.[14] The inoculations were given in graded doses starting with 0.5 ml. and increasing to 4.0 ml. Five days after the last inoculation, the rabbits were exsanguinated by cardiac puncture, the individual serums were heated for one half hour at 56 C., and stored at —40 C. until used. In the immunization treatment, a total of 10.395 mg. of stroma N was administered. Optimal amount of antibody for sensitization of the erythrocyte suspension was determined as described by Osler *et al.*[12]

*Method.*—The c′-fixation method employed has been described in detail by Osler *et al.*[12] and will be given only briefly here in order to point out the slight modifications introduced.

To varying dilutions of test serum (antibody) is added the appropriate constant c′ dilution followed by a constant dose of antigen as follows: Arithmetically progressively dilutions of antibody in 0.4-ml. amounts are added to the test tubes (13 by 100 mm.) which are in an ice bath. This is followed by 0.5 ml. of an ice-cold c′ dilution calculated to contain five 50 per cent hemolytic units (5 $c'H_{50}$). The antigen dilution in 0.4-ml. amounts is then added to the appropriate tubes. A sufficient quantity of veronal buffer to bring the total volume to 1.3 ml. is added to the control tubes, which contain only c′ plus antigen or c′ plus antibody. After thorough shaking, the tubes are put in the refrigerator at 2 to 5 C. for overnight storage. The sensitized cell suspension containing $10^8$ optimally sensitized cells, in a volume of 0.2 ml., is then added to the reaction mixtures immediately after they have been removed from the refrigerator. The resulting mixtures are incubated at 37 C. for 60 minutes, during which time the tubes are thoroughly and frequently shaken to prevent settling of the cells.

All tubes not showing complete lysis are centrifuged, and the degree of lysis is visually estimated and recorded as 0, 1, 2, 3, or 4 (*i.e.*, equivalent to no lysis, 25%, 50%, 75%, and 100% lysis, respectively). The end point is taken as the dilution of antiserum in which the lysis has been recorded as 2. When several antibody dilutions show this degree of lysis, the average of these dilutions is taken as the end point.

A c′ titration containing 1, 2, 3, 4, and 5 units of c′ is set up with each experiment in order to control the c′ dilution, conditions of overnight storage of the reaction mixtures, and the susceptibility of the sensitized sheep erythrocytes to immune lysis.

## RESULTS

*Determination of Antigen Dilution.*—To determine the concentration of antigen to be employed in these tests, block titrations were set up in which dilutions of antibody were combined with various dilutions of antigen in the presence of a constant dose of c′ (*i.e.*, 5 $c'H_{50}$). The protocol and results of a typical experiment (table 1) show that, with the antigen dilutions tested, there was no indication of a region of antigen excess. Further, there was no sharp lower limit of antigen concentration after which c′ fixation was not demonstrable. The magnitude of the fixation reaction was dependent on the amount of antigen employed as well as the antibody concentration.

Wallace, Osler, and Mayer [20] have shown that, for a single immune system, the optimal antigen dilution may vary with serums from different animals or even with serums from the same animal when they are taken after different courses of immunization. In the present work, it has not been possible to determine the optimal antigen dilution because of the anticomple-

mentary activity of the vesicular fluid. For the tests to be described in this work, therefore, the antigen has been used at the lowest dilution compatible with avoiding anticomplementary complications. Although the control tube containing the 1:40 dilutions of antigen showed complete lysis, the speed of lysis obtained with this antigen concentration was greatly retarded. For this reason, a 1:60 dilution of vesicular fluid was used.

*Fixation Time and Temperature.*—Throughout this work, the fixation period has been for 18 to 20 hours at 2 to 5 C. Preliminary tests had shown that a fixation period of one hour at 37 C. was not sufficient for maximal fixation. Typical results of a comparative titration of the same serum sample with these two fixation periods are shown (table 2).

The titer of this antiserum when assayed with overnight fixation at 2 to 5 C. was 1:1,340. For the shortened fixation period, the titer was found to be 1:70. This variation in titer appears to be due only to the difference in incubation periods, since all the reagents were the same in both tests. With serums of lower titer, there was no apparent fixation at 37 C. for one hour.

*Differences in Apparent Antibody Titer with Antigens from Different Sources.*—Since the virus antigens in general use in most diagnostic laboratories are prepared from infected tongue material, it was important to determine the comparative value of such antigens from this source with those prepared from infected vesicular fluids. The activity of virus from tissue culture was also assayed (table 3). Three block C′-fixing titrations were set up containing, as antigen, dilutions of *(a)* infected tongue epithelium, *(b)* tissue-culture virus, and *(c)* vesicular fluid.

Greater fixation was obtained with vesicular fluid than with either of the other two antigens, emphasizing the importance of the source of antigen for maximal reactions. Although complement-fixing antibodies were demonstrable with tongue-epithelium and tissue-culture antigens, the maximum antibody titers obtained with these antigens were only a fraction of the titer found with the vesicular fluid as antigen.

The reason for this great difference when employing these different antigens is not known. Although all three of these antigens have approximately the same infective titer,[9,19] it appears as if the 20 per cent tongue-epithelium and undiluted tissue-culture antigens contain less antigen active in C′ fixation than does the 1 per cent vesicular fluid. Therefore, the vesicular fluid may have a greater concentration of the noninfectious particles which, in addition to virus, are active in C′ fixation.[2,7] This point, however, will have to be proved by further quantitative studies.

*Appearance and Disappearance of Antibody in Infected Cows.*—Four cattle were

**TABLE 1—Block Complement-Fixation Titration with Varying Dilutions of Bovine Serum 13-2 vs. Varying Dilutions of Type C, Foot-and-Mouth Disease Vesicle Fluid**

| Antiserum dilution | Antigen dilution | | | | | |
|---|---|---|---|---|---|---|
| | 1:40 | 1:50 | 1:60 | 1:70 | 1:80 | V. B.* |
| 1:25 | 0 | 0 | 0 | 1 | 2 | 4 |
| 1:50 | 0 | 0 | 1 | 1 | 2 | 4 |
| 1:75 | 0 | 0 | 1 | 2 | 3 | 4 |
| 1:100 | 0 | 1 | 1 | 3 | 3 | 4 |
| 1:125 | 0 | 1 | 2 | 3 | 4 | 4 |
| 1:150 | 1 | 2 | 2 | 4 | 4 | 4 |
| 1:175 | 1 | 2 | 3 | 4 | 4 | 4 |
| 1:200 | 1 | 2 | 3 | 4 | 4 | 4 |
| 1:225 | 1 | 3 | 4 | 4 | 4 | 4 |
| 1:250 | 1 | 3 | 4 | 4 | 4 | 4 |
| 1:275 | 2 | 3 | 4 | 4 | 4 | 4 |
| 1:300 | 2 | 4 | 4 | 4 | 4 | 4 |
| V. B.* | 4 | 4 | 4 | 4 | 4 | 4 |

* Veronal buffer. The reactions, set up in the cold, contained 0.4 ml. of the designated antiserum dilutions, 5 C′H₅₀ in 0.5 ml. and 0.4 ml. of the designated antigen dilutions. Fixation was overnight at 2 to 5 C. Sensitized cells (0.2 ml.) were added, and the lytic period was for 60 minutes at 37 C. After centrifugation, the lysis was visually estimated and recorded as 0 = no lysis, 1 = 25 per cent, 2 = 50 per cent, 3 = 75 per cent, and 4 = complete lysis. (This procedure was also employed in the tests reported in tables 2 and 3.)

**TABLE 2—Comparison of Antibody Titers Obtained with Bovine Antiserum 31-5 vs. Type C, Foot-and-Mouth Disease Virus when Using Fixation Periods of (A) One Hour at 37 C. and (B) Overnight at 2 to 5 C.**

| | (A) Fixation for one hour at 37 C. | | | (B) Fixation overnight at 2 to 5 C. | | |
|---|---|---|---|---|---|---|
| Tube No. | Antiserum dilution | Lysis | V.B.* | Antiserum dilution | Lysis | V.B.* |
| 1 | 1:20 | 1 | 4 | 1:600 | 0 | 4 |
| 2 | 1:40 | 1 | 4 | 1:700 | 0 | .... |
| 3 | 1:60 | 1 | 4 | 1:800 | 0 | .... |
| 4 | 1:80 | 3 | .... | 1:900 | 0 | .... |
| 5 | 1:100 | 3 | .... | 1:1,020 | 1 | .... |
| 6 | 1:120 | 3 | .... | 1:1,120 | 1 | .... |
| 7 | 1:140 | 3 | .... | 1:1,200 | 1 | .... |
| 8 | 1:160 | 4 | .... | 1:1,280 | 2 | .... |
| 9 | 1:180 | 4 | .... | 1:1,400 | 2 | .... |
| 10 | 1:200 | 4 | .... | 1:1,600 | 3 | .... |
| 11 | 1:220 | 4 | .... | V.B.† | 4 | .... |
| 12 | 1:240 | 4 | .... | .... | .... | .... |
| 13 | V.B.† | 4 | .... | .... | .... | .... |

* V.B. = Antiserum with only veronal buffer and C′.
† V.B. = Antigen with only veronal buffer and C′.
The reactions, set up in the cold, contained 0.4 ml. of the designated antiserum dilutions, 5 C′H₅₀ in 0.5 ml. and 0.4 ml. of antigen diluted 1:60.

**TABLE 3—Block C′ Fixation Titrations with Type A-5 Antiserum 10-5 vs. Type A-5 Virus from (a) Infected Tongue Epithelium, (b) Tissue Culture, and (c) Vesicle Lymph**

| Antiserum dilution | Percentage of tongue epithelium | | | | | |
|---|---|---|---|---|---|---|
| | 20 | 10 | 7.4 | 5.0 | 4.0 | 3.3 |
| 1:50 | 0 | 0 | 0 | 0 | 1 | 1 |
| 1:100 | 0 | 1 | 1 | 2 | 2 | 4 |
| 1:200 | 4 | 4 | 4 | 4 | 4 | 4 |
| 1:300 | 4 | 4 | 4 | 4 | 4 | 4 |
| 1:400 | 4 | 4 | 4 | 4 | 4 | 4 |
| 1:500 | 4 | 4 | 4 | 4 | 4 | 4 |
| V.B.* | 4 | 4 | 4 | 4 | 4 | 4 |
| | Percentage of tissue-culture fluid | | | | | |
| 100 | 20 | 10 | 6.7 | 5.0 | 4.0 | V.B.* |
| 0 | 4 | 4 | 4 | 4 | 4 | 4 |
| 2 | 4 | 4 | 4 | 4 | 4 | 4 |
| 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| | Percentage of vesicle lymph | | | | | |
| | 2.0 | 1.6 | 1.4 | 1.2 | 1.0 | V.B.* |
| 1:600 | 0 | 0 | 0 | 0 | 0 | 4 |
| 1:700 | 0 | 0 | 0 | 0 | 1 | 4 |
| 1:800 | 0 | 0 | 0 | 1 | 1 | 4 |
| 1:900 | 0 | 0 | 0 | 1 | 2 | 4 |
| 1:1,000 | 0 | 0 | 1 | 1 | 2 | 4 |
| 1:1,100 | 0 | 1 | 2 | 2 | 3 | 4 |
| 1:1,200 | 1 | 1 | 2 | 2 | 3 | 4 |
| 1:1,300 | 1 | 2 | 3 | 3 | 4 | 4 |
| 1:1,400 | 1 | 2 | 3 | 4 | 4 | 4 |
| 1:1,500 | 2 | 2 | 4 | 4 | 4 | 4 |
| 1:1,600 | 3 | 4 | 4 | 4 | 4 | 4 |
| V.B.* | 3 | 4 | 4 | 4 | 4 | 4 |

* Veronal buffer. The reactions, set up in the cold, contained 0.4 ml. of the designated antiserum dilutions, 5 $C'H_{50}$ in 0.5 ml. and 0.4 ml. of the designated antigen dilutions. Fixation was overnight at 2 to 5 C.

used for each of the virus types, $O_1$, $A_5$, and $C_1$. Cows 1 and 2 were infected with type $O_1$ tissue-culture virus, while cows 3 and 4 received type $O_1$ infective bovine tongue epithelium virus. Cows 9 to 12 and 13 to 16 were infected with bovine tongue epithelium virus types $A_5$ and $C_1$, respectively.

With the exception of cow 1, which developed tongue lesions only, all animals showed generalization in all four feet. The results of the c′-fixation analyses with the serums from these animals are shown (graphs 1–3).

In the serums of the animals tested, it was possible to follow the course of the development and disappearance of antibody by direct c′ fixation. The curve relating antibody titer with postinfection time was qualitatively the same in each of the cattle, irrespective of the type of virus employed. There is an initial period of approximately four days during which no antibody is demonstrable. Antibody first becomes detectable on the seventh day after infection. (No tests were taken on the fifth and sixth days.) The titer increases rapidly on subsequent days, until a maximum is attained at two or three weeks postinfection. After the peak is reached, there is at first an immediate and rapid decrease in the antibody titer for about one week, followed by a gradually slower rate of disappearance for the next five weeks.

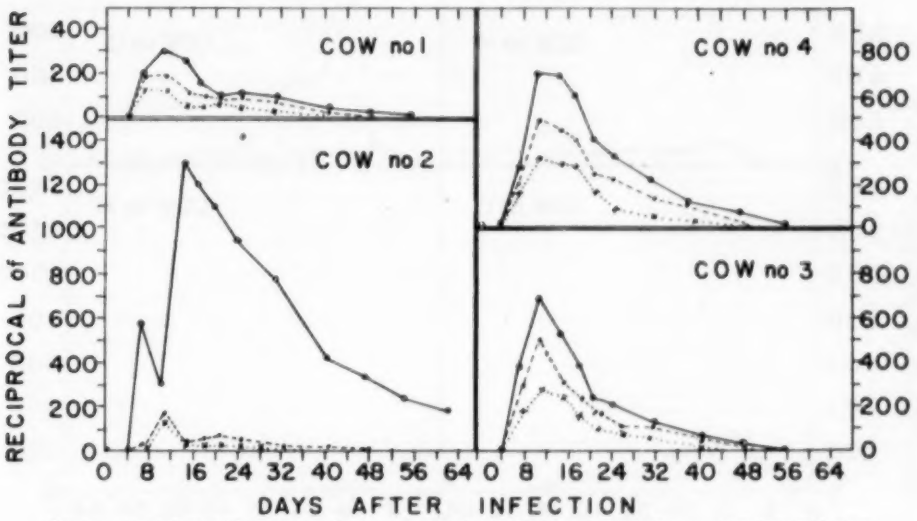
The curves obtained with the heterologous types of antigens (graphs 1–3), in addition to showing the magnitude of cross-reactivity of these serums, serve to establish the virus specificity of the reaction. Since all three antigens represent vesicular fluid pools from 20 to 30 animals, the only difference in their composition is in the virus-type particle and possibly in secondary antigens. In all assays, the virus had been used at the same dilution. If the c′-fixation reactions were due to antigenic constituents of the vesicular fluid other than the virus and specific secondary antigens, identical quantitative reactions would have been anticipated with all the antigens employed. The fact that this did not occur appears to confirm the specificity of the reaction.

## DISCUSSION AND SUMMARY

In the diagnosis of foot-and-mouth disease (FMD) and typing of the viruses, the direct complement (c′) fixation test is an important tool.

The two techniques most commonly used are those described by Traub and Mohlmann [18] and by Brooksby.[4] Both of these methods employ hyperimmune guinea pig serum as the antibody. These tests, therefore, necessitate tissue from the suspected animal that is either suitable for use as antigen in the c′-fixation test, or is infective when inoculated into a susceptible animal. Since suitable virus material exists for only a few days following infection, it can not always be obtained from field cases. Therefore, a complement-fixation test which utilizes bovine convalescent or immune serum has much practical significance.

The first practical solution of this problem was reported by Rice and Brooksby [16] and by Rice and Boulanger.[15] They developed an indirect c′-fixation method for detection of antibody in serums from cows infected with FMD. Antibody was detected in 30 of 34 cattle suspected of having had FMD. Two of 4 calves experimentally in-

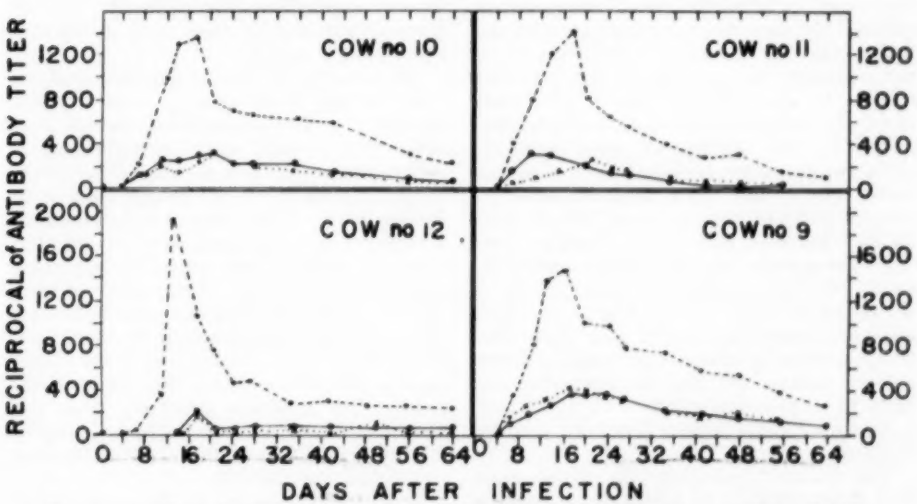



Key: o—o reactions with type $O_1$ antigen; . — . reactions with type $A_5$ antigen; . . . . reactions with type C antigen.

**Graph 1—Titer of complement-fixing antibody in cattle serum following experimental infection with type $O_1$ FMDV.**

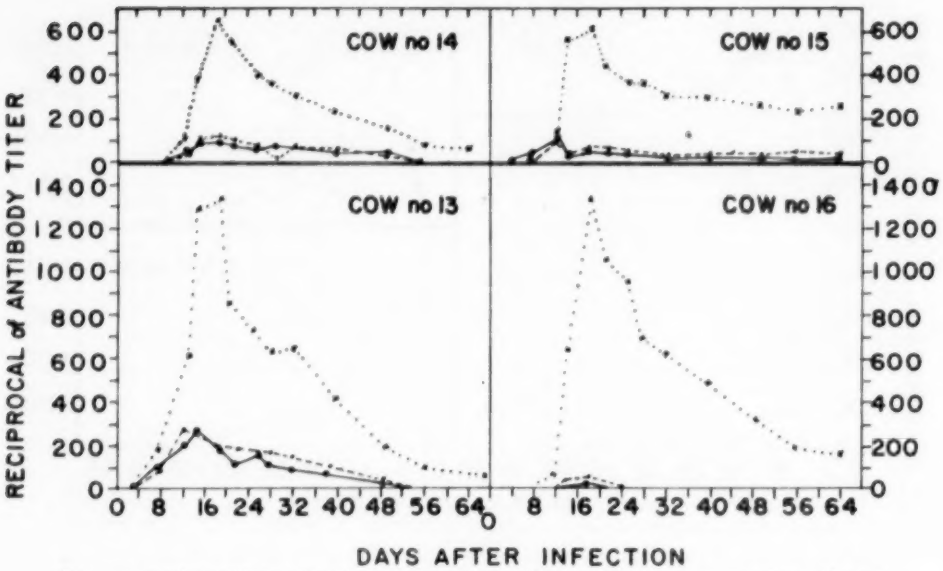
fected with foot-and-mouth disease virus (FMDV), showed increased inhibitory activity by the indirect c′-fixation technique, but only 1 showed weak fixation by the direct method. These authors used tongue epithelium and infected guinea pig plantar pads as antigen, with a fixation period of one hour at 37 C.

The direct method employed in this report was developed from the fundamental studies of Heidelberger, Mayer, Osler, and their co-workers,[10-12] and incorporates the




Key: . — . reactions with type $A_5$ antigen; . . . . reactions with type C antigen; o—o reactions with type $O_1$ antigen.

**Graph 2—Titer of complement-fixing antibody in cattle serum following experimental infection with type $A_5$ FMDV.**




Key: . . . . reactions with type C antigen; o—o reactions with type $O_1$ antigen; . — . reactions with type $A_5$ antigen.

**Graph 3—Titer of complement-fixing antibody in cattle serum following experimental infection with type C FMDV.**

following procedures for increased sensitivity and reproducibility: (1) The reaction mixtures have been maintained at pH 7.4 with veronal buffer to provide a medium for optimal c′ function. (2) Calcium (0.00015 M) and magnesium (0.0005 M) have been incorporated in the buffer since these ions have been shown to be necessary for optimal c′ fixation and lysis. (3) Amboceptor and c′ have been carefully standardized spectrophotometrically. (4) The number of sheep erythrocytes employed has been kept at a constant value ($10^8$), and in a uniform state of susceptibility to immune lysis. (5) Gelatin (0.5%) has been added to the buffer to reduce nonspecific loss of antigen, antibody, or c′. (6) The reaction mixtures have been set up in the cold. (7) Antibody dilutions have been made in parallel with close dilution intervals in order to accurately define the end points. (8) The fixation period has been 18 to 20 hours at 2 to 5 C. (9) Fluid from FMD tongue lesions has been used as antigen.

The curves describing the appearance and disappearance of antibody in serums of cattle convalescent from FMD infection are similar to those described for other animal species with other antigens.[6,17] There is an initial induction period of approximately seven days after infection, during which time no antibody is detectable. This is followed by a rapid rise in antibody to a peak in about two to three weeks. Thereafter, the antibody titer decreases, first rapidly, then more slowly over a protracted period.

The results of the c′-fixation analyses with serums from cow 2 were atypical (graph 1). The course was normal until after the seventh day postinfection, at which time there was a rapid drop in the antibody titer to almost 50 per cent of its former level. This drop was followed by a renewed and increased antibody production, reaching a titer approximately twice that observed in any of the other animals of this group. A possible explanation for this atypical reaction might be that, after seven days, this animal may have developed a severe viremia. This, then, could reduce the apparent antibody titer and also would act as a secondary antigenic stimulus, resulting in the observed increase in antibody production.

It will be important to determine what, if any, relationship exists between the antibody titer determined by this method and the state of immunity of the animals. The

peak of antibody titer, as measured in these tests, corresponds well with that of protecting antibody.[7] However, it has been found with other virus antigens that the serums from convalescent animals retain their neutralizing activity long after their ability to fix $c'$ has disappeared.

The technique has been used to follow the course of antibody appearance and disappearance in cattle experimentally infected with types $O_1$, $A_5$, and C FMDV.

Studies on the demonstration of antibody in vaccinated cattle by direct $C'$-fixation are now in progress. The results will be given in a subsequent publication.

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# Critical Tests with Piperazine-Carbon Disulfide Complex (Parvex) Against Parasites of the Horse

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NUMEROUS investigations in recent years indicate the efficacy and safety of the piperazine compounds as anthelmintic agents in man and domestic animals. In the horse, most of the studies have been concerned with the use of piperazine adipate, and there is agreement on the high degree of efficiency against *Parascaris equorum* and the small strongyles.[1,7,8,10,12] In addition, there is evidence of removal activity on the order of 50 to 60 per cent against the causative agent of verminous aneurysms, *Strongylus vulgaris*,[1,7] and 80 per cent against adult pinworms, *Oxyuris equi*.[1]

More recently, the effectiveness of the citrate, phosphate, and carbodithioic acid (carbon-disulfide) derivatives were reported[8,9] to compare favorably with the adipate. Fecal egg counts following treatment indicated that ascarids had been eliminated and strongyles markedly reduced. In addition, the carbon disulfide compound was found to produce this action at a lower dose level (60% piperazine base equivalent) than the other compounds, and there was removal activity against bots.

At the outset, the piperazine-carbon disulfide compound would appear to offer special value in the horse where the activity of the carbon disulfide component against bots and ascarids would be combined with the activity of the piperazine base against ascarids, strongyles, and pinworms. Thus, this agent might have a polyvalent action against the common parasites of horses hitherto unavailable in a single compound.

The present investigation was undertaken to evaluate by the critical testing method the removal activity of the piperazine-carbon disulfide compound against the several types of parasites common to horses in this region.

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## METHODS

Five of 7 test animals were of mixed breeding; the mare and 1 yearling (620) were Thoroughbreds. All of the parasitic infections were naturally acquired, and only the mare had been given recent anthelmintic treatment; piperazine citrate (220 mg./kg.) 26 days previously. Yearling 620 had received periodic treatments with carbon disulfide and phenothiazine as a suckling and weanling colt.

The only special preparation of the animals was the withholding of hay and grain on the morning the drug was administered and for four hours after treatment, when hay was offered. After this, the regular ration of hay and oats was given. A suspension* containing 250 mg. of betaine of 1-piperazine carbodithioic acid per cubic centimeter was administered by stomach tube. All of the animals were treated with the same lot of the suspension except 2 (W62 and Y624) which received a later preparation.

The critical testing method was used to determine the removal activity against bots, ascarids, large and small strongyles, and mature and immature pinworms. In addition, a series of quantitative egg and larval examinations were made on the feces to compare the infections before and after treatment.

A complete collection was made of the feces between treatment and necropsy six days later. At each 24-hour interval, the feces was weighed and thoroughly mixed. For the small strongyle and immature pinworm estimations, replicated 10-Gm. subsamples comprising 1 per cent of the 24-hour feces were carefully examined in shallow glass dishes under x 3 magnifying lamps. The remainder of the 24-hour fecal collection was carefully picked through twice, before and after washing (20-mesh screen), to recover the bots, ascarids, large strongyles, and adult pinworms. Similar fecal examinations were made on Y623 and Y624 for the 24-hour period immediately preceding treatment.

At necropsy, a complete examination was made of the gastrointestinal tract contents and linings

* Parvex is a trademark of the Upjohn Co. for the suspension of betaine of 1-piperazine carbodithioic acid, 250 mg./cc.

for the larger parasites. A sampling procedure similar to that described for the feces was employed on the cecal, ventral colon, and dorsal colon contents. Replicated 50-cc. samples of the respective contents, comprising 2 per cent of the original contents, were examined to estimate the number of small strongyles and immature pinworms. A preliminary digestion of the glandular mucosa of the stomach in artificial gastric juice was used to facilitate the search for *Trichostrongylus axei* and *Habronema muscae*, which were estimated from the counts on samples constituting 10 per cent of the total digest and contents. A similar digestion of the anterior portion of the small intestinal wall was made in Y623 and Y624 to determine the presence of *Strongyloides westeri*. No attempt was made to detect the passage of *T. axei* or *S. westeri* in the feces of any of the horses.

A modification of the Stoll flotation method was employed for the worm egg counts (e.p.g.), using 10 Gm. of feces diluted to 100 ml.

Cultures for the larval counts (l.p.g.) were prepared from 100-Gm. samples of feces, which were placed in a Baermann apparatus after storage for one week at room temperature, and the number of larvae was estimated by dilution counts. The larvae of *T. axei* and *S. vulgaris* were differentiated from the other strongyle larvae. In the mare, differentiation was also made of the larvae of *Strongylus edentatus*.

## RESULTS

The critical test data on the seven trials with piperazine-carbon disulfide compound are summarized (table 1).

Two species of bots were encountered. Removal activity against *Gastrophilus intestinalis* was variable and ranged from 17 to 100 per cent, including 2 animals which had heavy infections. Although infections of *Gastrophilus nasalis* were lighter, none was found at necropsy after doses of 150 mg./kg. and above. Passage of bots in the feces started during the 24- to 48-hour post-treatment interval, continued for the six-day period, and some were recovered at necropsy from the contents of the cecum and colons of all but 2 animals. The bots recorded (table 1) as recovered at necropsy include only those found alive and attached in their normal locations, *i.e.*, in the stomach (*G. intestinalis*) and at the pylorus (*G. nasalis*).

Complete removal activity was observed against ascarids in these trials. Immature forms were encountered in 2 animals and mature ascarids were harbored by 6. The bulk of the ascarids were passed in the feces during the second and third days after treatment, and a few ascarid fragments were found as late as the fourth day in 3 animals. Considerable disintegration of the worms voided after the second day was noticed and they were characterized

**TABLE 1—Critical Test Data on the Efficacy of Piperazine-Carbon Disulfide Compound Against Parasites of the Horse**

| Horse | Wt. (kg.) | Dose (mg./kg.) | Bots | | Ascarids (*P.e.*) | | Pinworms (*O.e.*) | Strongylus | | | | Stomach worms | | | Tapeworms |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *G.i.* | *G.n.* | Mature | Immature | Mature | Immature | *S.e.* | *S.ed.* | *S.eq.* | Small | *T.a.* | *H.m.* | *S.w.* | *A.m.* |
| **PARASITES ELIMINATED IN FECES AFTER TREATMENT** | | | | | | | | | | | | | | | | |
| W62 | 199 | 75z | 8 | 2 | 48 | 343 | 184 | x | x | x | x | x | x | x | x | x |
| Mare L.L. | 555 | 100 | 20 | 11 | x | x | x | x | x | x | x | x | x | x | x | x |
| Y621 | 236 | 100 | 80 | 5 | 11 | 0 | 179 | 860 | 59 | 0 | 0 | 42,185 | x | x | x | x |
| Y622 | 243 | 150 | 199 | 2 | 47 | 2 | 0 | 5,290 | 60 | 1 | 0 | 24,300 | x | x | x | x |
| Y620 | 277 | 150 | 2 | 0 | 1 | 0 | 0 | 0 | 124 | 0 | 0 | 94,045 | x | x | x | x |
| Y623 | 290 | 200 | 217 | 43 | 19 | 0 | 0 | 4,875 | 5 | 5 | 0 | 8,270 | x | x | x | x |
| Y624 | 297 | 200z | 21 | 0 | 0 | 0 | 0 | 2,425 | 112 | 38 | 0 | 57,120 | x | x | x | x |
| **PARASITES RECOVERED AT NECROPSY 6 DAYS AFTER TREATMENT** | | | | | | | | | | | | | | | | |
| W62 | ..... | ..... | 0 | 0 | 0 | 0 | 103 | x | x | x | x | x | x | x | x | x |
| Mare L.L. | ..... | ..... | 99 | 1 | x | x | x | x | x | x | x | x | 12,200 | 0 | x | 0 |
| Y621 | ..... | ..... | 19 | 3 | 0 | 0 | 61 | 11,960 | 32 | 0 | 0 | 1,490 | 1,420 | 80 | x | 0 |
| Y622 | ..... | ..... | 227 | 0 | 0 | 0 | 0 | 28,370 | 119 | 56 | 0 | 2,970 | 20 | 0 | x | 0 |
| Y620 | ..... | ..... | 1 | 0 | 0 | 0 | 0 | 400 | 85 | 15 | 0 | 5,275 | 190 | 10 | x | 0 |
| Y623 | ..... | ..... | 208 | 0 | 0 | 0 | 0 | 12,270 | 8 | 8 | 0 | 130 | 130 | 30 | 0 | 1 |
| Y624 | ..... | ..... | 0 | 0 | 0 | 0 | 0 | 13,760 | 116 | 94 | 2 | 3,090 | 230 | 140 | 15 | 0 |
| **PERCENTAGE OF EFFICACY** | | | | | | | | | | | | | | | | |
| W62 | ..... | ..... | 100 | 100 | 100 | 100 | 64 | .... | .... | .... | .... | .... | ..... | ..... | ..... | ..... |
| Mare L.L. | ..... | ..... | 17 | 92 | .... | .... | .... | .... | .... | .... | .... | .... | ..... | ..... | ..... | ..... |
| Y621 | ..... | ..... | 81 | 63 | 100 | .... | 75 | 7 | 65 | .... | .... | 97 | ..... | ..... | ..... | ..... |
| Y622 | ..... | ..... | 47 | 100 | 100 | 100 | .... | 16 | 34 | 2 | .... | 89 | ..... | ..... | ..... | ..... |
| Y620 | ..... | ..... | 66 | .... | 100 | .... | .... | 0 | 53 | 0 | .... | 95 | ..... | ..... | ..... | ..... |
| Y623 | ..... | ..... | 51 | 100 | 100 | .... | .... | 28 | 89 | 38 | .... | 98 | ..... | ..... | ..... | ..... |
| Y624 | ..... | ..... | 100 | .... | .... | .... | .... | 15 | 43 | 29 | 0 | 95 | ..... | ..... | ..... | ..... |

x = no observation made; z = new suspension: *G.i.* = *Gastrophilus intestinalis*; *G.n.* = *Gastrophilus nasalis*; *P.e.* = *Parascaris equorum*; *O.e.* = *Oxyuris equi*; *S.v.* = *Strongylus vulgaris*; *S.ed.* = *Strongylus edentatus*; *S.eq.* = *Strongylus equinus*; *T.a.* = *Trichostrongylus axei*; *H.m.* = *Habronema muscae*; *S.w.* = *Strongyloides westeri*; *A.m.* = *Anoplocephala magna*.

**TABLE 2—Egg and Larval Count Data on the Efficacy of Piperazine-Carbon Disulfide Complex Against Parasites of the Horse**

| | Eggs per gram of feces | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Parascaris equorum* | | | Strongyles | | | *Trichostrongylus axei* | | |
| Horse | No. before treatment* | No. after treatment† | Change (%) | No. before treatment* | No. after treatment† | Change (%) | No. before treatment* | No. after treatment† | Change (%) |
| W62 | 360.0 | 0 | −100 | 1,880.0 | 480.0 | −75 | 0 | 0 | — |
| Mare L.L. | 0 | 0 | — | 255.0 | 65.0 | −74 | 33.0 | 32.0 | −3 |
| Y621 | 40.0 | 0 | −100 | 840.0 | 70.0 | −92 | 0 | 10.0 | + |
| Y622 | 250.0 | 0 | −100 | 740.0 | 55.0 | −93 | 0 | 0 | — |
| Y620 | 0 | 0 | — | 525.0 | 30.0 | −94 | 2.0 | 0 | −100 |
| Y623 | 140.0 | 0 | −100 | 650.0 | 15.0 | −98 | 0 | 10.0 | + |
| Y624 | 0 | 0 | — | 1,515.0 | 245.0 | −84 | 0 | 0 | — |
| | Larvae per gram of feces | | | | | | | | |
| | *Strongylus vulgaris* | | | Other strongyles | | | *Trichostrongylus axei* | | |
| W62 | 0 | 12.0 | + | 720.0 | 260.0 | −64 | 0 | 0 | — |
| Mare L.L. | 39.0 | 9.0 | −77 | 244.0 | 64.0 | −74 | 28.0 | 31.0 | +11 |
| Y621 | 63.0 | 19.0 | −70 | 818.0 | 48.0 | −94 | 23.0 | 25.0 | +9 |
| Y622 | 100.0 | 67.0 | −33 | 508.0 | 111.0 | −78 | 1.0 | 1.0 | 0 |
| Y620 | 103.0 | 21.0 | −80 | 276.0 | 18.0 | −93 | 0.6 | 0.3 | −50 |
| Y623 | 10.0 | 3.5 | −65 | 403.0 | 8.0 | −98 | 0 | 0.5 | + |
| Y624 | 125.0 | 13.0 | −99 | 1,260.0 | 182.0 | −86 | 2.0 | 0.3 | −85 |

* Average of four to seven counts during seven-day pretreatment interval. † Average of two to three counts during fourth to sixth day post-treatment.

by a partial to complete brownish discoloration and extensive fragmentation. These changes made an accurate count and the stage of maturity difficult to determine; however, the numbers given are regarded as conservative estimates.

Two animals were harboring adult *O. equi* and the observations credit the drug with removing 64 and 75 per cent, respectively. Adult pinworms were present in the feces during the first 12 hours after dosing and they continued to be passed for four and seven days, respectively. Heavy infections of immature pinworms were present in all but 1 yearling, but removal activity was variable and of a relatively low order. Elimination of these forms was recorded throughout the post-treatment observation period in Y621 and Y623. A definite peak elimination period during the 24- to 48-hour interval was noted in 3 (Y622, Y623, and Y624) at the higher dose levels.

Of the three species of large strongyles, *S. vulgaris* was predominant and was most effectively removed. The level of activity varied from 34 to 65 per cent. Increasing the dose did not enhance the activity against this species. Only limited activity against *S. edentatus* was observed and this came at the highest dose level, 200 mg./kg. A light infection of *Strongylus equinus* was encountered in 1 animal and no drug activity was found. Characteristically, the bulk of the large strongyles was recovered in the feces during the 24- to 48-hour interval after treatment, although some were found as late as the fourth day in 3 animals.

All strongyles, other than the three Strongylus species, are recorded as small strongyles (table 1). A consistently high level of activity against these forms was observed, ranging from 89 to 98 per cent. The size of the infections varied from light (8,270) to heavy (100,220), and the greatest numbers were removed 24 to 48 hours after treatment. Some small strongyles appeared in the feces during the first 24 hours after dosing, and elimination continued for varying periods up to the sixth day in 1 yearling.

Infections of *T. axei* were found in all but 1 animal at necropsy and were characteristically light in the yearlings and moderately heavy in the mare. Small numbers of *H. muscae* were present in the stomachs of 4 animals. A few *S. westeri* were recovered from the small intestine of 1 of the 2 yearlings examined at necropsy. One *A. magna* was recovered from 1 yearling.

Worm egg and larval counts were made in conjunction with these critical tests (table 2).

After treatment, the weanling and all 3 yearlings showing ascarid eggs became negative and striking reductions in strongyle e.p.g. occurred in the other animals. The *T. axei* e.p.g. in the yearlings were of a low order and variable, irrespective of

treatment, and the higher count observed in the mare was not changed.

The numbers of strongyle larvae were also markedly reduced after treatment (table 2). Reductions (64 to 98%) were consistently more marked in the "other strongyle" group, which included all species except *S. vulgaris*. The number of *S. vulgaris* larvae was reduced to a lesser extent (33 to 90%). Although the absolute number of *S. vulgaris* larvae was reduced by treatment, there was a proportionate increase in this species, except in 2 animals.

Larvae of *S. edentatus*, included in the other strongyle group (table 2), were differentiated before and after treatment of the mare. They showed a decline from an average of 88 to 52 (41%) per culture. The average make-up of the post-treatment cultures on this mare consisted of 9 *S. vulgaris* and 52 *S. edentatus* larvae out of the total of 73 strongyle l.p.g.

Only 2 animals showed an appreciable number of *T. axei* larvae in the cultures and no essential change was associated with treatment.

No untoward effects, ascribable to a toxic manifestation of the drug, were observed other than a transitory period during the first 48 hours in which the feces was softened in the 2 animals receiving the highest dose level.

## Discussion

Although the activity of piperazine-carbon disulfide compound against bots was definite, the removal activity was well below the high level previously reported [5,14,15] for carbon disulfide. The greater activity against *G. nasalis* may be accounted for by its location at the pylorus which favored contact with the drug. The less effective action against *G. intestinalis* could be due to an incomplete dissociation of the compound or the failure of the liberated carbon disulfide to reach the bots in sufficient concentrations as suggested earlier.[8] The 100 mg./kg. dose was equivalent to the 6 dr. per 1,000 lb. of body weight of carbon disulfide ordinarily employed. The lack of activity at the 200 mg./kg. level suggests that the limiting factor, yet to be determined, is operating at a surprisingly high level. However, 100 per cent activity against the relatively light infections of *G. intestinalis* in both trials with the newer preparation of the suspension was more favorable and in accord with the unpublished findings of others.[13]

Elimination of the bots was prolonged more than the discharge of the worms, the great bulk of which were eliminated in 72 hours. However, the recovery of large numbers of bots in the feces over the six-day post-treatment period and in the cecal and colon contents at necropsy is not at variance with previous observers.[5,14] Hall *et al.*[5] indicated that "bots may be passed for ten days and others still may be present in the large intestine 17 days after treatment" with carbon disulfide. While this slow elimination is difficult to explain in terms of a prolonged action of the drug or stasis in the gastrointestinal tract, it could indicate the immediate removal from the normal location (*i.e.*, stomach) to a less favorable environment in the large intestine to which the bots are unable to adapt themselves and are gradually eliminated.

The complete elimination of ascarid infections with the drug at the 75 mg./kg. rate, which is below the previously reported effective level,[8,9] is of special interest because it indicates that the piperazine-carbon disulfide compound is effective at about one-half the dose level found for the other piperazine compounds in the horse, possibly because of the presence of two components in the carbon disulfide derivative which are active against the ascarids. Additional unpublished findings [2] in field tests are in support of this lower effective dose level for this preparation. To date, doses of 75 mg./kg. administered by stomach tube have been uniformly consistent in completely eliminating ascarid eggs and markedly reducing strongyle eggs in feces one week after treatment of Thoroughbred sucklings, weanlings, and yearlings. Additional critical tests to determine the minimal effective dose against ascarids in the horse would be of interest. The drug would appear to be more efficient when given by stomach tube, as to the horse, than when given in the feed, as to swine, where the minimal effective rate was 125 mg./kg.[8,11]

In general, the efficacy of piperazine-carbon disulfide complex against both small and large strongyles is comparable to other work with piperazines, and of a similar order to that reported [3,4] for phenothiazine in therapeutic doses of 30 Gm. for adult horses.

The high level of activity against the small strongyles from doses of 100 mg./kg. in these critical tests was forecast in earlier reports [8,9] in which this dose level effectively reduced strongyle egg counts. Less effective action was indicated in the single trial at 75 mg./kg., but this observation is at variance with field trials [2] in which this dose has characteristically reduced strongyle e.p.g. on the order of 90 per cent. Thus, in accord with the observations on the ascarids, the piperazine-carbon disulfide complex appears to give as satisfactory removal of small strongyles at approximately one-half to two-thirds the dosage (piperazine base equivalent) reported [1,8] for other piperazine derivatives.

The large strongyles were not so effectively removed as the small strongyles; however, an appreciable level of action against *S. vulgaris* was evidenced and is similar to that reported for the adipate.[1,8] More complete action against this species was not associated with the dose increases in these trials. Limited activity against *S. edentatus* appeared only at the highest dose level tested. The number of *S. equinus* encountered was insufficient to provide a satisfactory test of activity.

These results indicated a much higher level of activity against the mature pinworms than the immature fourth-stage larval forms. Unfortunately, mature worms were present in only 2 animals, and definite conclusions can not be drawn on this limited evidence; however, the activity was observed at the lower dose levels and is similar to that reported for the adipate.[1] Although the efficacy against the immature pinworms was of a low order, definite activity of the drug was shown in the peak elimination periods following treatment. This elimination could not be ascribed to the normal tendency of a continuous natural passage of the fourth-stage larvae experienced by others [1] and also noticed in 2 animals in the present series.

There was no indication of activity against *T. axei*. Reductions in egg and larval counts were not associated with treatment, and specimens of this species were recovered at necropsy in all animals, 1 of which presented a moderately heavy infection.

Definite evidence of removal activity against *H. muscae* was not obtained. Carbon disulfide has been regarded [10] as an effective treatment, but none of these forms was detected in the feces after treatment and, at necropsy, two-thirds of the animals showed infections, ranging from light to moderate.

Comparison between the worm collection data and the egg and larval counts presents several interesting points. The marked reductions in strongyle egg counts in general reflected the high level of activity determined for the small strongyles but were misleading as an index of action against the large strongyles, which were much less effectively removed. The reductions in the larvae of *S. vulgaris* also tended to give an estimate of activity which was higher than that indicated by worm collections. This apparent discrepancy could be accounted for by a selective removal of more mature individuals. The increase in the proportion of *S. vulgaris* among the strongyle larvae after treatment is in accord with the worm collection data and reflects the more effective removal of the small strongyles than of the large strongyles. The egg and larval counts on *T. axei* were in agreement and failed to indicate any activity against this species.

## SUMMARY

Seven critical tests were made with a suspension of piperazine-carbon disulfide complex (Parvex) in horses at dosages of 75, 100, 150, and 200 mg./kg. of body weight. Complete removal of both mature and immature ascarids and nearly complete removal (89 to 98%) of the small strongyles were observed. Large strongyles were less effectively removed (34 to 65% for *Strongylus vulgaris;* 0 to 38% for *Strongylus edentatus*). The efficacy against bots ranged from 17 to 100 per cent for *Gastrophilus intestinalis* and 63 to 100 per cent for *Gastrophilus nasalis*. Activity against immature pinworms was of a low order (0 to 28%), while limited evidence indicated a more complete action (64 to 75%) against the adult forms. There was no evidence of activity against the stomach worms *Trichostrongylus axei* and *Habronema muscae*.

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# Equine Ringworm with Special Reference to *Trichophyton Equinum*

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TRADITIONALLY, the various dermatophyte species that cause animal ringworm have been named for their host. Thus, the dermatophyte isolated from dogs was named *Microsporum caninum* or *Microsporum canis*. The specific name *Microsporum felineum* was given to the cat parasite, and *Microsporum equinum* to that from horses. A morphologically similar fungus isolated from human beings with ringworm, however, was named *Microsporum lanosum* because of its woolly texture. Further studies indicated that these organisms were one and the same.[1-5] Today, most mycologists consider that *M. canis* Bodin, 1902, is the valid name for the Microsporum commonly isolated from infected dogs, cats, and human beings.

In the case of the Trichophytons, a similar situation existed. A fungus isolated from ringworm of the dog was named *Trichophyton caninum* by Matruchot and Dassonville,[6] a cat isolate was designated *Trichophyton felineum* by Blanchard,[7] and a culture isolated from a horse was named *Trichophyton equinum* by Gedoelst.[8] Later workers agreed that many of the trichophytons isolated from animals were identical to *Trichophyton mentagrophytes* (Robin) Blanchard, 1896, which had been isolated from human cases of ringworm. However, one important exception is *T. equinum* which many workers have accepted as a species distinct from *T. mentagrophytes*.

Sabouraud[9] was careful to point out that there were two Trichophyton species commonly involved in ringworm infections of horses. One of these he described as producing a flat, white to creamy, powdery to granular, rapidly growing colony. He called this fungus *Trichophyton granulosum*. Many mycologists believe that this corresponds closely to the granular variety of *T. mentagrophytes* which is recognized today as a common cause of animal ringworm. The other Trichophyton which Sabouraud designated as an important cause of equine ringworm was *T. equinum*, a fungus first described by Matruchot and Dassonville in 1896[10] and named by Gedoelst in 1910.[8] This fungus was characterized by the development of a white, fluffy colony with brilliant yellow pigmentation at the edges and reverse side of the colony.

In the Mycology Unit of the Communicable Disease Center, recent interest in animal ringworm and its transmission to man has stimulated a study of *T. equinum* in an effort to determine whether it is a distinct species, and whether it is associated almost exclusively with horses. *Trichophyton equinum* has not been recognized as such in this country. Conant *et al.*[11] list it as a synonym of *T. mentagrophytes;* however, the name appears frequently in the European literature.

Our first experience with *T. equinum* occurred several years ago when numerous Trichophyton species were obtained from various culture collections for nutritional studies. One strain listed as *T. equinum* (Baudet) had been obtained from the Centraal Bureau voor Schimmelcultures, Holland. Studies indicated that this isolate would not grow on a vitamin-free medium unless supplied with nicotinic acid. This vitamin could be replaced with nicotinic amide or the amino acid, tryptophane. Since this was the first species among the pathogenic fungi known to have a requirement for nicotinic acid, this finding was reported.[12] Unfortunately, the culture was almost pleomorphic, and its original appearance could not be determined. It was assumed from the name *T. equinum* that it had been isolated from a horse. It was not known at the time whether the requirement for nicotinic acid was characteristic of all isolates of *T. equinum*.

Recently, during the animal ringworm survey being conducted by this laboratory,[13] we had an opportunity to study a number of fresh isolates of Trichophyton obtained from ringworm-infected horses in the United States and Canada. A similar survey * in England made it possible for

* From the Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare, Atlanta, Ga.

* Conducted by Drs. P. K. C. Austwick and G. C. Ainsworth.

**TABLE 1—Data from Animal Ringworm Survey**

| Series No. | Material received for study | Source of material and brief clinical data | Final identification |
|---|---|---|---|
| 1 a, b | Horse hair | Clinical material from 2 race horses involved in ringworm epizootic at Atlantic City race tracks, New Jersey, 1953.[20] | *Trichophyton equinum* |
| 2 | Culture | Culture isolated from horse at Ontario Veterinary College, 1953.* | *T. equinum* |
| 3 | Horse hair | Clinical material from saddle horse, Florida, 1953. Scaling and eczematoid lesions on head, neck, and flank.† | *T. equinum* |
| 4 | Horse hair | Clinical material from a female adult Tennessee Walking horse, Ohio, 1954. Eczematoid lesions on flank, abdomen, and leg.† | *T. equinum* |
| 5 | Horse hair | Clinical material from race horse, New Jersey, 1955. Two-year-old Thoroughbred horse with scaling lesions on neck, chest, and shoulder.† | *T. equinum* |
| 6 | Dog hair | Clinical material from a 4-month-old, male Doberman Pinscher dog, Kentucky, 1955. Generalized loss of hair and scaling. The dog was born and raised in a rural area where direct or indirect contact with horses could have occurred.† | *T. equinum* |
| 7 | Horse hair | Pooled clinical material from 2 colts (approximately 2 years old), Colorado, 1955. Lesions on backs of both animals.† | *T. equinum* |
| 8 | Culture identified as *T. equinum* | From a pony, Perth, Scotland, 1953. Disease soon spread to 5 other ponies. Lesions described as circular swellings which later become moist, eczematous, and depilated; some suppuration. Lesions largely in saddle and girth areas. (Received infected hairs also.)‡ | *T. equinum* |
| 9 | Culture identified as *T. equinum* | From a horse in a riding stable following introduction of a new horse, Reading, England, 1953. Lesions similar to above, occurring largely in saddle and girth areas. After 2 months, almost every horse in the stable had the disease. (Infected hairs also received.)‡ | *T. equinum* |
| 10 | Culture identified as *T. equinum* | From a case of ringworm in a riding school near that above.‡ | *T. equinum* |
| 11 | Culture identified as *T. equinum* | From ringworm of a horse, England. Lesions on saddle and flank areas.‡ | *T. equinum* |
| 12 | Culture identified as *T. equinum* | From ringworm of a horse, England. Lesions on saddle and flank areas. (Infected hairs also received.)‡ | *T. equinum* |
| 13 | Culture listed as *T. equinum* strain Baudet | Central Bureau of Fungus Cultures, Delft, Holland, 1946. Isolated from horse. | *T. equinum* |
| 14 | Culture listed as *T. equinum* | From a race horse involved in an epizootic. A caretaker also contracted the infection.[20]‡ | *T. equinum* |
| 15 | Culture listed as *T. equinum* | Sabouraud Collection. Culture isolated in 1930.§ | *T. equinum* |
| 16 | Culture identified as *T. mentagrophytes* | Isolated from a horse at Ontario Veterinary College, 1955.* | *Trichophyton mentagrophytes* var. *granulosum* |
| 17 | Culture identified as *T. mentagrophytes* | Isolated from a Thoroughbred adult horse, Sevenoaks, England, 1955. Showed small lesions with loss of hair and crust formation in area of stifle joint.‡ | *T. mentagrophytes* var. *granulosum* |
| 18 | Horse hair | Clinical material from an adult horse pastured with cattle showing ringworm lesions, Michigan, 1953. Thick brownish crusts on neck and back.† | *Trichophyton verrucosum* var. *verrucosum* |
| 19 | Culture identified as *T. discoides* | From a pony, Wimborne, England, 1956. Lesions not numerous but up to 1½ inches in diameter and irregular in shape. Hair at edges lifted off easily, leaving raw surfaces. Hard dry crusted lesions also observed.‡ | *T. verrucosum* var. *discoides* |

**TABLE 1 (Continued)—Data from Animal Ringworm Survey**

| Series No. | Material received for study | Source of material and brief clinical data | Final identification |
|---|---|---|---|
| 20 | Culture listed as *T. equinum* | American Type Culture Collection. From a horse, 1945.[33] | *T. verrucosum* var. *album* |
| 21 | Culture identified as *M. equinum* | From a horse in Glasgow, Scotland, 1953. Wood's light showed a few fluorescent hairs. No other clinical details. (Hairs also received.)‡ | *Microsporum canis* |
| 22 | Culture identified as *M. equinum* | From a horse in North Wales, England.‡ | *M. canis* |
| 23 | Culture identified as *M. equinum* | From a horse in England.‡ | *M. canis* |
| 24 | Culture identified as *M. equinum* | From a horse, England. (Hairs also received.)‡ | *M. canis* |
| 25 | Culture labelled *M. equinum* | Sabouraud Collection. Culture isolated in 1930.§ | *M. canis* |
| 26 | Horse hair | From Standardbred horse 20 months old. Lesions over rump, croup, and under the mane with scaling and loss of hair.† | *Microsporum gypseum* |

* From Mr. J. B. Fischer, Ontario Department of Health, Toronto.
† From the Communicable Disease Center Animal Ringworm Survey.
‡ From Dr. P. K. C. Austwick, Veterinary Laboratory, New Haw, Weybridge, England.
§ From Dr. J. E. Mackinnon, Institute of Hygiene, Montevideo, Uruguay.

us to study a number of dermatophyte strains recently isolated from horses in that country. In addition, a number of strains listed as *T. equinum* and *M. equinum* were obtained from various culture collections in different parts of the world.

For purposes of comparison, several other dermatophyte species isolated from horses have been studied. These are: *T. mentagrophytes* var. *granular (T. granulosum, Trichophyton gypseum); Trichophyton verrucosum (Trichophyton faviforme, Trichophyton discoides, Trichophyton album, Trichophyton ochraceum); M. canis (M. equinum, M. felineum, M. lanosum);* and *M. gypseum (Achorion gypseum, Microsporum fulvum).*

## MATERIALS AND METHODS

Twenty-five strains of dermatophytes isolated from horses and one strain from a dog are included in this study. The original identification of these strains and their sources as well as our final identification based on the results of the studies described below are listed (table 1).

1) *Clinical Data and Study of Clinical Materials.*—Whenever the data were available, a description of the lesions on the horses, the number of animals involved, and the occurrence of human contagion were compiled. Direct examination of clinical materials, hairs, and skin scrapings was carried out whenever possible. Measurements of arthrospores and photographs of infected hairs were made in order to compare the manner of the hair invasion and particularly the size of the spores.

2) *Morphological Studies of Cultures.*—Determination of gross colony characteristics was made on Sabouraud dextrose agar. Microscopic morphology was studied on a wide variety of mediums including Sabouraud dextrose agar, wort agar, potato dextrose agar, and rice grains.

3) *Nutritional Studies of Cultures.*—All strains were tested for their ability to grow on a vitamin-free casein agar and on the same medium with nicotinic acid added. A group of strains, which responded to nicotinic acid, was tested for ability to utilize nicotinic amide or l-tryptophane. Strains which did not grow on the vitamin-free casein agar and which did not respond to nicotinic acid were tested for their ability to utilize thiamine or a combination of thiamine and inositol.

4) *Special Physiological Studies.*—Representatives of each type of organism were tested for their ability to perforate hair *in vitro*. This was done in accordance with the method described by Ajello and Georg.[34]

Guinea pig inoculations were made with a typical *T. equinum* strain and a typical *T. mentagrophytes* strain isolated from horses, to compare the lesions produced and to determine whether the morphological and physiological characteristics of these fungi would remain stable upon repeated animal passage.

Typical *T. mentagrophytes* strains from human

sources were serially grown and transplanted on horse hairs in water over a period of eight months in an effort to determine whether any morphological or physiological changes occurred after growth under these conditions. These strains were also grown on a nicotinic acid medium for the same length of time.

5) *Genetic Studies.*—Attempts were made to obtain biochemical mutants from an apparently autotrophic strain of *T. mentagrophytes* var. *granular* obtained from ringworm of the horse, as well as from an equine strain which was morphologically similar but showed a partial requirement for nicotinic acid. Methods used consisted of the selection of colonies from serially streaked plates and delayed enrichment as described by Lederberg and Lederberg.[15]

## RESULTS

Following a study of the available clinical data as well as a review of cases reported in the literature, it was concluded that in general a similar clinical picture was produced by all the agents that cause equine ringworm. Minor variations in the severity of lesions, contagiousness of the infections, and the manner of hair invasion do occur. These will be discussed separately under each fungous species.

The classic description of ringworm of the horse by Matruchot and Dassonville in 1898[10] can not be improved upon. These authors had occasion to observe and study an epizootic of ringworm which occurred among the horses of an artillery regiment in France. Since their findings correspond rather closely to the findings in all types of ringworm seen in this series, their description will be quoted in part.

> The animals presented a number of ringworm plaques. Isolated lesions were scattered over the hindquarters and shoulders, others were grouped together on the posterior part of the saddle area and some were confluent. The isolated lesions first appeared as swellings and were perceptible by touch before they could be seen. Some days later the plaques became visible because the hairs were matted and disordered in these areas. At this time the hairs came out easily. Slight traction detached most of the infected hairs in a single bloc, the remainder being held at their base by a scaly crust. The skin in the area was at first moist and had a rosy or greyish cast. Soon afterwards the plaque itself became dry, becoming powdery or granular and dark slate grey in color. The lesions enlarged through the loss of peripheral hairs. They were most numerous in the saddle and girth area and formed large patches by their confluence.

In a few of the cases from which the strains in this series were derived, the lesions had developed thick crusts from which pus and serum could be expressed. However, this type of reaction was the exception, with dry scaling lesions being the rule. There was evidence of severe itching, particularly where heavy crusts had formed. The severe itching seen in ringworm of the horse has led to the use of the term "girth itch" for this condition.

Although the gross clinical picture of equine ringworm was similar in all cases, microscopic examination of infected hairs revealed that the manner of hair invasion, particularly the size of the arthrospores produced, varied considerably with the fungous species involved. Only by obtaining the invading fungus in culture, however, was the diagnosis of ringworm confirmed and the specific identity of the fungous agent determined.

Each of the five fungi involved in ringworm of the horse will be discussed separately with regard to *(a)* prevalence, *(b)* special factors of the disease, *(c)* the manner of hair invasion, and *(d)* morphological and *(e)* physiological characteristics of the fungi.

The basis for considering the fungus known as *T. equinum* as a distinct species that commonly causes ringworm infections of the horse will be discussed following the presentation of our observations on the clinical materials and cultures obtained for this study.

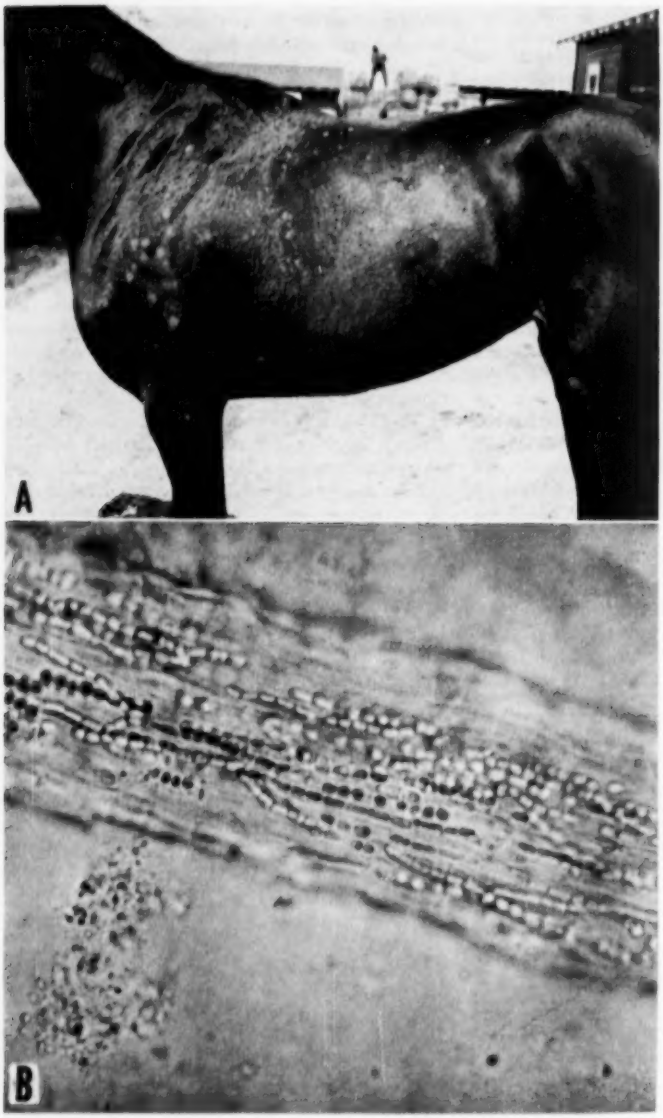
### I. TRICHOPHYTON EQUINUM

*a) Prevalence of Infections.*—This fungus appears to be the most common cause of equine ringworm in the United States, Europe, and South America. Of 26 infections recorded in this study, 15 (57.7%) were due to this organism. The other 11 represented four other species. The literature indicates that *T. equinum* has caused much ringworm in horses in the past. Matruchot and Dassonville first described the fungus from horses in France in 1898. Sabouraud stated in 1908[16] that it was common in horses in the Paris area. Brocq-Rousseu *et al.*[17] reported *T. equinum* in 20 of their 100 cases of ringworm of the horse in 1927; Neefs and Gillain[18] found it in 27 of 321 infected horses, in a remount depot in Belgium in 1928; and Lebasque[20] found it in 28 of 100 cases in 1933. Mackinnon[19] describes an epizootic among horses in Uruguay due to *T. equinum* in

1936 (*see* culture 14, table 1). The epizootic of ringworm in horses reported by Negroni in Argentina in 1941 [20] was probably due to this fungus too.

Ainsworth and Austwick's recent survey of animal mycoses in Britain [21] has revealed that *T. equinum* infection in the horse is common in that country. They described a number of epizootics which rapidly spread from stable to stable. The incubation period appeared to be about three weeks.

A number of strains of this fungus were isolated in the United States and Canada during our animal ringworm survey. The majority came from isolated cases of equine




—*William S. Miller, Haddonfield, N. J.*

**Fig. 1—(A) Ringworm of the horse due to Trichophyton equinum.**

**B) Horse hair infected by T. equinum. Ectothrix type of parasitism. x 522.**

ringworm. The strains from New Jersey (No. 1 and 5) came from an epizootic, described by Batte and Miller, [22] which developed in race horses (fig. 1A) in several Atlantic Coast stables. These horses are continuously moved as the racing season progresses southward in the fall and northward in the spring. This fungus was first isolated from this epizootic in 1953 and again in 1955. Prolonged treatment appeared to be effective in controlling the spread of the disease in the treated animal. However, reinfection and new cases arose due to the introduction of infected animals into a stable. Colts and yearlings were the most susceptible, but animals of all ages became infected. It is probable that infected hairs which remained on brushes, combs, and saddle gear were a source of infection. Fungi on hair have been found to remain viable for a period of a year and a half when kept in a dry test tube in the laboratory. Walker [23] has had similar experience.

Human contagion has occurred in a number of the epizootics, but the number of cases was apparently low.[6, 9, 17, 19] Sabouraud [9] described 4 cases of *T. equinum* infections in adult men, all on bearded areas of the face. In 3 cases, there was considerable tissue reaction and the development of kerions with many parasitized hairs. The fourth case, which presented an early lesion, showed only a small raised erythematous area. The hairs were at an early stage of parasitism. Ainsworth and Austwick [21] did not report human contagion from equine ringworm. In our present survey, we have not observed cases of human infection due to this fungus.

*b) Manner of Hair Invasion by Trichophyton Equinum.*—The manner of hair invasion by *T. equinum* was adequately described by Matruchot and Dassonville.[10] The hairs examined in the various cases seen in our series conformed to their description. The infected hairs revealed large masses of oval to round spores at their bases with the spores often completely filling the root sheath. On the upper portion of the hair, chains of spores were irregularly dispersed on the surface with the majority of these chains running parallel to the length of the hair. In some areas, a complete sheath of spores was present. Where the spores had become dislodged, mycelium and some spore chains were seen in the interior of the hairs. The diameter of the arthrospores varied from 3.5 to 8.0 $\mu$ with an average size of 5.8 $\mu$. This type of hair invasion is most correctly described as "ectothrix with medium-sized spores in chains." The size of the spores is intermediate between that of *T. mentagrophytes* (3.0 to 5.0 $\mu$) and the extremely large-spored ectothrix Trichophyton, *T. verrucosum* (5.0 to 10.0 $\mu$). Sabouraud described the hair invasion of *T. equinum* as "ectothrix megaspore" in contrast to the "microide" type produced by *T. mentagrophytes;* however, he pointed out that the spores were not as large as those of the "faviform species" *(T. verrucosum).* Figure 1B illustrates a hair infected by *T. equinum.*

*c) Morphological Characteristics of Trichophyton Equinum.*— (1) Gross Colony Characteristics.—The characteristics of this fungus are described as they developed on Sabouraud dextrose agar. The fungus grows rapidly forming a white, downy to fluffy colony 20 cm. in diameter by the seventh day. The surface is at first flat but, by the end of the second week, it may show irregular heaping and shallow folding (fig. 2A). At the edge of the colony, there is usually a fringe of submerged growth which is obvious because of its brilliant yellow-orange pigmentation. The reverse side of the colony is at first a lemon yellow, but soon becomes bright yellow-orange.† As the culture ages, this pigment gradually changes to a deep rose red.‡

All of the recently isolated strains corresponded closely to the above description and agreed with the description given by Matruchot and Dassonville for this organism. One of the strains from the "Sabouraud collection" (No. 15 of this series) did not develop yellow pigmentation. The reverse side of this colony was at first light pink and later became deep rose red. The strain from the Centraal Bureau voor Schimmelcultures in Holland (No. 13 in this series) was extremely fluffy and showed no pigmentation. Since both of these strains had been in culture for many years and were partially pleomorphic, they had undoubtedly lost their original pigmentation.

2) Microscopic Appearance of the Culture of *Trichophyton Equinum.*—All strains, including those recently isolated, sporulated poorly on Sabouraud dextrose agar. The branching, septate mycelium, about 2.0 $\mu$ in diameter, bore a few to many microconidia. These were single-celled, slightly clavate to elongate spores borne along the sides of the mycelium. Some were attached directly and others were on short, delicate sterigmata. They were easily broken from the mycelium. Average dimensions were 3.0 to 4.0 $\mu$ by 2.0 to 3.0 $\mu$ (fig. 2B). The amount of sporulation was greatly increased on potato-dextrose agar or on wort agar. On wort agar, microconidia were numerous and often nearly spherical in shape. They occurred not only along the sides of the mycelium but in dense grapelike clusters. A few macroconidia were observed in some of the strains on this medium. These were variable in size and shape but were generally slightly clavate. They had thin, smooth

† Ridgeway, "Orange Chrome," plate II, color No. 11; Munsell notation, 2.5 YR 6.0/15.0.

‡ Ridgeway, "Scarlet Red," plate II, color No. 3; Munsell notation, 5R 4.4/16.0.

walls and were composed of three to four cells. Poorly developed spirals were seen in one strain. Chlamydospores were numerous in old cultures.

The microscopic picture of the strains studied in this series appeared to be similar to those for this species described by Matruchot and Dassonville, with the exception that these authors did not observe macroconidia. Mackinnon, however, reported finding macroconidia and spirals in the strains he isolated from horses in Uruguay.[10]

*d) Physiological Characteristics of Trichophyton Equinum.*—All of the strains studied which had the above-described morphology were found to have a complete requirement for nicotinic acid. None of the strains would grow on $NH_4NO_3$ or casein (acid-hydrolyzed) vitamin-free mediums, but they all grew well on similar mediums containing nicotinic acid (2 mg./ml.). This requirement could be replaced by equimolar amounts of nicotinic amide or relatively large amounts of l-tryptophane. The method for performing such nutritional tests has been described previously.[12] One strain (No. 15 in this series) which had been obtained from the Sabouraud Collection showed only a partial requirement for nicotinic acid. As this strain had been in the culture collection at least since 1930, it is possible that it had undergone some change since its original isolation. The culture was nearly sterile and did not produce the yellow pigment characteristic of this species. The other nearly pleomorphic strain from the Dutch collection (No. 13), which had been in culture at least since 1946, still exhibited a complete requirement for nicotinic acid.

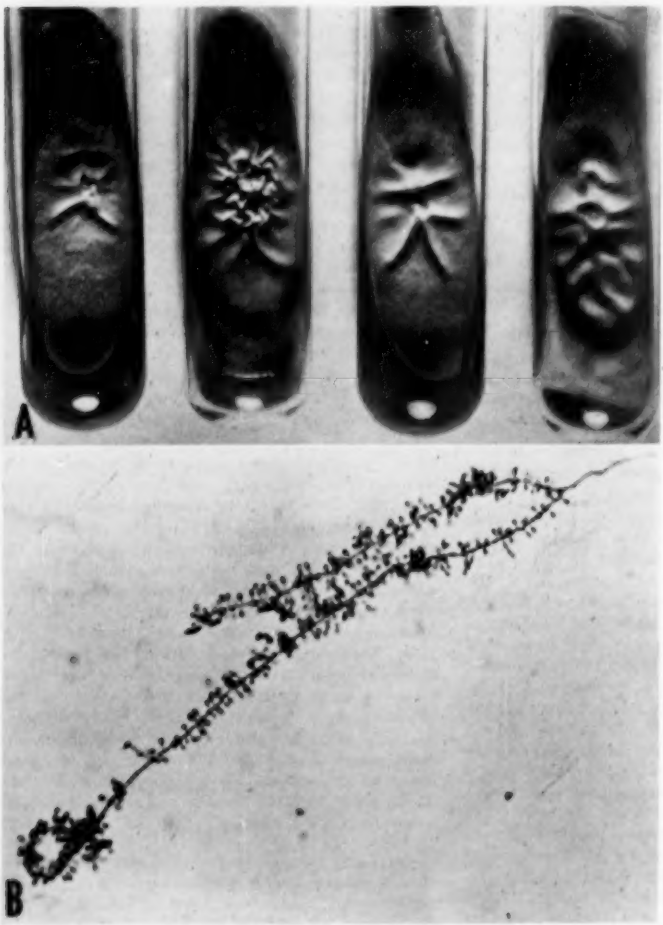



Fig. 2—(A) Trichophyton equinum. Variation in colony morphology.

B) Trichophyton equinum. Microscopic morphology from Sabouraud dextrose agar. Microconidia borne on sides of the mycelium. x 522.

## II. Trichophyton Mentagrophytes (Var. Granulosum)

*a) Prevalence of Infection.*—Although *T. mentagrophytes* has often been mentioned in the literature as an important cause of ringworm of horses, it appears to be a much more common cause of ringworm in other animal species. In our current survey of animal ringworm, this fungus has been isolated only once from horses; however, we have obtained 18 isolates from dogs, two from cats, six from chinchillas, and 42 from wild animals (13 mice, 18 rats, and 2 opposums).

From a historical point of view, *T. mentagrophytes* was the first dermatophyte species described as the cause of ringworm of the horse (Sabouraud, 1893).[24] This fungus was apparently a common cause of ringworm of the horse and of extensive epizootics in cavalry regiments at that time. An outbreak in the Sudan, in 1906, lasted nearly a year with 800 infections observed.[25] By 1908, Sabouraud [16] recorded that in the district of Paris this type of equine ringworm had become more rare and *T. equinum* more common. In the epizootics reported by Neefs and Gillain in 1931,[18] of 321 cases of ringworm in horses, none were due to *T. mentagrophytes*, while Brocq-Rousseu *et al.*[17] reported only 2.5 per cent, and Lebasque [26] found only exceptional cases due to this fungus. Catanei, however, recorded an epizootic in Algeria due to *T. mentagrophytes* in 1939.[27] In the present series, only 2 cases of ringworm in horses due to this fungus are recorded.

*b) Clinical Appearance of Hair Infected by Trichophyton Mentagrophytes.*—Clinical material was not available from the 2 equine cases in this series. One of the cultures (No. 16) was inoculated into a guinea pig, and infected hairs from this animal were studied. The picture of the hair invasion was similar to that seen in other infections due to *T. mentagrophytes* and corresponded closely to the description given by Sabouraud.[24] Actually, the picture is similar to that produced by *T. equinum* except that the size of the arthrospores is smaller, ranging between 3.0 and 5.0 $\mu$ and, in general, the spores are more uniform in size. Sabouraud spoke of the hair invasion by *T. mentagrophytes* as "ectothrix microide" and of the invasion by *T. equinum* as "ectothrix megaspore."

*c) Morphological Characteristics of Trichophyton Mentagrophytes var. Granular.*—The morphological characteristics of *T. mentagrophytes* var. *granular* are well known and need not be given in detail here. A brief description is included in table 2 for purposes of comparison with *T. equinum*. The two strains isolated from horses in this series (No. 16 and 17) were characteristic of this species both in gross and microscopic morphology.

*d) Physiological Characteristics of Trichophyton Mentagrophytes.*—Nutritional studies with this fungus by Robbins and Ma [20] indicate that this organism is autotrophic for vitamins. In the course of our routine nutritional studies, we have tested 25 strains of the granular variety of *T. mentagrophytes*. All of these grew well on casein vitamin-free medium. Twelve of these strains have been tested for stimulation by nicotinic acid. To date, only strain 17 which was isolated from a horse has shown any stimulation by this vitamin. Although this strain grows well on vitamin-free medium, a greater amount of growth occurs when nicotinic acid is added.

## III. Trichophyton Verrucosum

*a) Prevalence of Infection.*—In spite of the fact that one of the first isolations of the fungus now known as *T. verrucosum* was from an outbreak of ringworm involving 40 horses (and 9 human beings),[29] this fungus has rarely been reported as causing ringworm in horses. *Trichophyton verrucosum* is the common cause of ringworm in cattle in this country and other countries. In the course of our animal ringworm survey, *T. verrucosum* has been frequently isolated from ringworm in cattle, from an outbreak of ringworm among donkeys, and once from a burro and a horse. Ainsworth and Austwick,[21] who found this fungus to be a common agent of ringworm of cattle in England, also isolated it from a horse. In both of these equine cases, the horses had been pastured with ringworm-infected cattle.

*b) Clinical Appearance of Hair Infected by Trichophyton Verrucosum.*—There is little difference of opinion on the mode of the hair invasion by this organism. In general it has been described as a large-spored ectothrix or ectothrix megaspore, but the size of the spores is considerably greater than those of *T. equinum* which has been given this same descriptive term. In *T. verrucosum*, the size of the arthrospores ranges between 5.0 and 10.0 $\mu$ in diameter.

*c) Cultural Characteristics of Trichophyton Verrucosum.*—The gross morphology of this fungus has been described in another publication.[30] A brief description is presented (table 2). The high variation seen in the colonial form leads to the recognition of several varieties. The two isolated from horses in this series represented the varieties *discoides* and *verrucosum*. The *discoides* variety isolated in England has changed upon subculture to a typical *album* variety.

**TABLE 2—Diagnostic Characteristics of the Agents of Equine Ringworm**

| | *Trichophyton equinum* | *Trichophyton mentagrophytes* | *Trichophyton verrucosum* | *Microsporum canis* | *Microsporum gypseum* |
|---|---|---|---|---|---|
| Characteristics of infected hair. | Ectothrix. Sheath or isolated chains of spores (3.5 to 8.0 μ) lying on surface of hair. Mycelium seen in interior of hair. No fluorescence under Wood's lamp. | Ectothrix. Sheath or isolated chains of spores (3.0 to 5.0 μ) lying on surface of hair. Mycelium seen in interior of hair. No fluorescence under Wood's lamp. | Ectothrix. Sheath or isolated chains of large spores (5.0 to 10.0 μ) lying on surface of hair. Mycelium seen in interior of hair. No fluorescence under Wood's lamp. | Ectothrix. Sheath of small spores (2.0 to 3.0 μ) in mosaic. Completely surrounds hair at base. Mycelium seen in interior of hair. Fluoresce a bright yellow-green under Wood's lamp. | Ectothrix. Large spores in chains or irregular masses on surface of hairs (5.0 to 8.0 μ). Mycelium seen in interior of hair. No fluorescence under Wood's lamp. |
| Colonial morphology. | Growth rapid. Flat but develops irregular folds. Usually with a border of fringed, submerged growth. Surface downy to fluffy. White. Submerged growth and pigment on reverse a brilliant yellow-orange. | Growth rapid. Flat or heaped and irregularly folded. White to cream, powdery or granular surface. Pigment on reverse: yellow to rose to deep red-brown. | Growth very slow. May not appear until 10 to 14 days. Colony usually small, heaped, and folded. Surface white to grayish. Moist and smooth or slightly velvety. Growth more rapid at 37 C. or upon the addition of thiamine to the medium. Pigment on reverse: none. | Growth very rapid. Flat. Regular border. Surface white to yellowish tan. First fine, silky aerial growth and later fluffy with woolly areas. Pigment on reverse: bright yellow. Some strains lack the yellow pigment on reverse. | Growth very rapid. Flat. Irregular border. Surface cinnamon to light tan. Coarsely granular. Develops tufts of white fluffy growth. Pigment on reverse: tan. |
| Microscopic morphology. | Sabouraud dextrose agar.<br><br>Microconidia (single-celled spores): few to many, generally thin and elongate, 2.0 to 3.0 μ, occurring along sides of mycelium.<br><br>Macroconidia (large septate spores): very rare, irregular in size and shape, thin, smooth walls, and 2 to 3 cells.<br><br>Spirals: rare, poorly formed. | Sabouraud dextrose agar.<br><br>Microconidia (single-celled spores): numerous, usually nearly round, occur along sides of mycelium and in clusters.<br><br>Macroconidia (large septate spores): few to many, usually club-shaped and have thin, smooth walls and 3 to 5 cells.<br><br>Spirals: numerous in some strains. | Sabouraud dextrose agar.<br><br>Microconidia (single-celled spores): rare or absent. May be numerous on thiamine-enriched mediums.<br><br>Macroconidia (large septate spores): absent.<br><br>Mycelium: thin with irregular swellings and many chlamydospores (large double-walled resting spores). | Sabouraud dextrose agar.<br><br>Microconidia (single-celled spores): few or absent.<br><br>Macroconidia (large septate spores): very numerous, spindle-shaped with thick rough walls and 6 to 10 cells. | Sabouraud dextrose agar.<br><br>Microconidia (single-celled spores): rare or absent.<br><br>Macroconidia (large septate spores): very numerous, elliptical with thin, rough walls and 3 to 4 cells. |
| Special nutritional requirements. | Nicotinic acid. | None. | 90% of isolates require thiamine plus inositol. 10% require thiamine only. | None. | None. |

*d) Physiological Characteristics of Trichophyton Verrucosum.*—Nutritional studies of this fungus have been reported.[29-32] In the course of our routine nutritional studies, we have tested 92 strains of *T. verrucosum*. Of these, 78 strains (85%) require two vitamins, thiamine and inositol, for growth while 14 strains (15%) require only thiamine. One strain has been reported to require thiamine, inositol, and pyridoxine.[31]

In this series, the two strains isolated from horses were both found to require thiamine and inositol.

Since it had a complete requirement for thiamine and inositol, one strain obtained from the American Type Culture Collections labelled "*T. equinum*" also fell into this group. The original morphology was not observed, since this strain was pleomorphic when received. From Hoerlein's description of this strain,[33] as well as the results of our nutritional tests, it would appear that this strain should be classified as *T. verrucosum*.

## IV. Microsporum Canis

*a) Prevalence of Infection Among Horses.—Microsporum canis* infections in horses were first described by Bodin in 1896 [29] under the name of "*M. audouini* var. *equinum*." The name *M. equinum* was given to this fungus by Guéguen in 1904.[34] This species was differentiated from *M. canis* by early authors largely on the basis of the smaller size of the macroconidia. The identity of this fungus with *M. canis* was suggested by Conant [4] in 1935 and was later substantiated by a statistical analysis of spore size in the genus Microsporum by this same author.[5]

*Microsporum canis* is apparently a common cause of ringworm of the horse. Brocq-Rousseu *et al.*[17] found it the cause of 72.5 per cent of equine ringworm in army regiments in France and Algeria in 1928; Bergner [35] reported it in a German army transport unit involving 600 horses in 1942; Lebasque [26] found it in 60.0 per cent of the ringworm of horses in Algeria and Iran in 1934; Neefs and Gillain [18] reported it in 248 of 321 ringworm-infected horses; and Catanei [27] reported an epizootic in a garrison in Algeria in 1939. A single case was reported from the United States by Hoerlein in 1945.[33] Human contact cases were often observed.

To date, we have not isolated this fungus from horses; however, we have been able to study a number of recent isolates from the survey being conducted in England.

*b) Clinical Appearance of Hair Infected by Microsporum Canis.*—All authors agree that the infected hair is one of the typical ectothrix Microsporum type with small spores in mosaic. The average spore size is 2.0 to 3.0 $\mu$. Fluorescence of infected hairs under a Wood's lamp has been occasionally seen in ringworm of the horse.

The infected hairs furnished for study by Austwick were identical to those commonly seen in ringworm of the dog or cat.

*c) Microscopic Characteristics of Microsporum Canis.*—These have been well described and are generally well known. A brief description of this species is included in table 2.

The four recently isolated strains studied in this series (No. 21 to 24) fell well within the range of morphological variations seen in the *M. canis* cultures commonly isolated from cat and dog ringworm cases seen at our laboratory.

*d) Physiological Characteristics of Microsporum Canis.*—As far as we know, nutritional requirements of this particular species have not been thoroughly studied. No specific nutritional requirement has been reported. All of the strains studied in this series grew well on casein vitamin-free medium and were not stimulated by nicotinic acid.

## V. Microsporum Gypseum

*a) Prevalence of Infection.—Microsporum gypseum* ringworm of animals has not been recorded frequently in the past. A review of the literature by Ajello [36] indicates that up to 1935 only 61 such reports had been made. In one instance, 40 cases had been recorded in a single group of horses.[18]

The first known human case caused by this fungus was reported by Bodin in 1907.[37] Because of the heavily crusted "cuplike" lesions which he thought were similar to those seen in human favus due to *Achorion schoenleini (Trichophyton schoenleini)*, he placed the fungus in the genus Achorion as *A. gypseum*. Later workers found that cuplike crusts were not a constant finding in either human or animal infections and, in fact, simple scaling with loss of hair or suppurative kerionic lesions were sometimes seen. Not until 1928 did Guiart and Grigorakis [38] place Bodin's fungus in the genus Microsporum on the basis of its morphological characteristics.

Epizootics in horses, attributed to this fungus, have been reported by Neefs and Gillain [18] in 1928 (40 of 321 cases) and by Bergner,[35] in 1942, in a group of 600 infected German army transport horses. No human cases were reported in these epizootics.

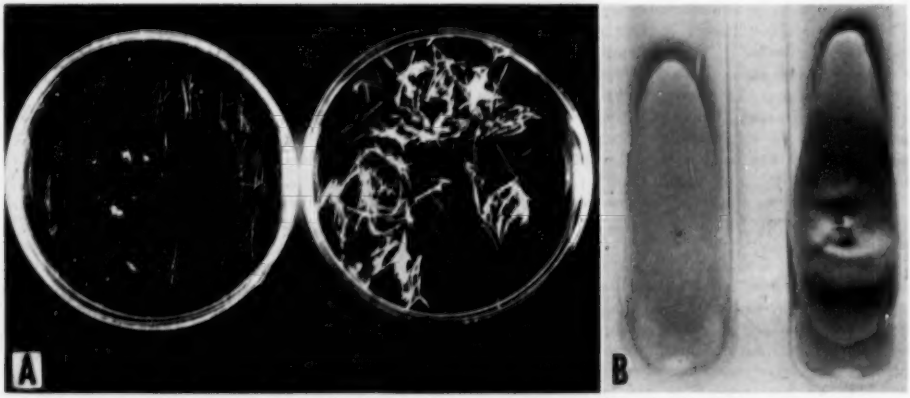



**Fig. 3—(A) No growth of Trichophyton equinum on human hairs in distilled water (Petri dish on left) compared to luxuriant growth on horse hairs (right).**

**B) Nutrition test showing requirement for nicotinic acid by Trichophyton equinum. Tube on left: $NH_4NO_3$ dextrose agar; right: $NH_4NO_3$ dextrose agar with added nicotinic acid.**

In the present series, only one instance of *M. gypseum* infection in a horse was seen. Since it had not been previously reported in this country, this equine case has been described in detail elsewhere.[39]§ This type of ringworm is apparently common in the dog. To date, we have isolated 64 strains from ringworm-infected dogs, two from cats, two from wild mice, and two from wild rats.

Recent studies by Ajello[36] have shown that *M. gypseum* is a common soil saprophyte. It seems odd that more infections in man and animals are not seen. Actually, it is difficult to produce experimental infections with this fungus. The organism appears to be essentially a soil saprophyte which only occasionally produces infections. Although a few instances of contagion from animals to man have been recorded, in most instances of human infection such evidence is lacking. It seems probable that both man and animals acquire their infections directly from the soil.

*b) Clinical Appearance of Hair Infected by Microsporum Gypseum.*—In all of the observed cases in which infected hairs were available for study, the fungus appeared as a large-spored ectothrix. Masses of the arthrospores occur both on skin scrapings and on the surface of hairs. These spores are highly irregular in size and shape but, in general, they are extremely large, ranging from 5.0 to 8.0 $\mu$ with an average spore size of 6.0 $\mu$. On the hairs, the arthrospores seldom occur as a distinct sheath but are irregularly massed over its surface. Linear chains running along its length can usually be detected in these masses. The interior of the hair may be filled with branching mycelium. The infected hair, therefore, does not resemble hairs infected by other Microsporum species such as *M. canis*. In contrast to *M. canis* infections, the hair infected by *M. gypseum* is not usually fluorescent under the Wood's lamp.

*c) Morphological Characteristics of Microsporum Gypseum.*—The culture from the equine case of *M. gypseum* ringworm was characteristic of the fungus as usually described. A brief morphological description is given (table 2).

*d) Physiological Characteristics of Microsporum Gypseum.*—Nutritional studies of *M. gypseum* have been reported by Johnson and Grimm.[40] This fungus appears to be autotrophic for the various vitamins. When this strain was isolated from ringworm of the horse it showed no special nutritional requirements.

## DISCUSSION

Five different dermatophyte species have been found associated with ringworm infections of the horse. In order of frequency, these are: (1) *T. equinum*, (2) *M. canis*, (3) *T. mentagrophytes* var. *granular*, (4) *T. verrucosum*, and (5) *M. gypseum*.

*Trichophyton equinum* appears to be closely related to *T. mentagrophytes* but it is distinct in colonial morphology, nutritional requirements, and size of arthrospores produced on infected hair. *Trichophyton equinum* appears to be consistently associated with ringworm of the horse. Rare infections have been reported in human contacts and in a dog.

§ Since this manuscript was prepared, the authors have observed an epizootic of *M. gypseum* ringworm involving 8 horses in one herd. It will be described (in J.A.V.M.A.) at a later date.

It is interesting to postulate that nicotinic acid-requiring mutants of *T. mentagrophytes* survived while growing as parasites on the horse. *Trichophyton mentagrophytes* and *T. equinum* found in ringworm of the horse suggests this possibility. One of the morphologically typical *T. mentagrophytes* var. *granular* strains isolated from ringworm of the horse (No. 17) was found to be stimulated by nicotinic acid. This finding suggests that intermediate forms may exist.

The question arose as to whether horse hairs offered any special nutritional environment which might allow for the selection of a nutritionally deficient organism. Several specimens of hair from horses, human beings, and other animal species (cat, dog, rabbit, guinea pig, monkey, donkey, and mule) were obtained for comparative studies. Typical isolates of *T. mentagrophytes* var. *granular* and of *T. equinum* were grown on autoclaved pieces of these various types of hair in sterile water. *Trichophyton mentagrophytes* grew on all of the hair specimens. However, it was remarkable that the amount of growth on horse hair and hair of related species was two or three times as great as that obtained on human or nonequine hair (fig. 3A).

*Trichophyton equinum* acted quite differently. This species did not grow on human hair under these conditions. However, it grew luxuriantly on horse, donkey, and mule hair.

Since *T. equinum* has been shown to have a complete requirement for nicotinic acid, it appeared probable that equine hair furnished available nicotinic acid or nicotinic acid precursors while nonequine hair did not. To determine if this was the differentiating factor, nicotinic acid was added in the amount of 20 $\mu$g. per milliliter of water in which the nonequine hair had been placed. Under this condition, *T. equinum* grew as well on the nonequine as on the equine hair.

It would appear that *T. equinum* produces ringworm almost exclusively in equine species because its nutritional requirements are adequately met in this environment. The fungus is aptly named. The fact that infections of man and dogs do occasionally occur, and that this type of ringworm can be induced experimentally in the guinea pig, indicates that the fungus can survive on the dermal tissues of these hosts. However, infections are short lived and further transmission usually does not occur.

Since horse hair appears to furnish nutrition not available in human hair, it was important to determine whether growth of the common varieties of *T. mentagrophytes*, such as variety *granular* from human suppurative ringworm, and the downy variety from human tinea pedis, might be altered in any way by serial transplantation and growth on horse hairs in water. Such serial transplants were made every two weeks for a period of eight months. Serial transplants on nicotinic acid-enriched casein agar (2 mg./ml.) were made during the same period of time. Other than a tendency to become pleomorphic, no changes either morphological or physiological were observed in these strains.

There was also speculation as to whether these varieties of *T. mentagrophytes* produced biological mutants (specifically mutants requiring nicotinic acid) under normal conditions. Accordingly, a number of apparently autotrophic strains of *T. mentagrophytes* were serially streaked on agar plates and a large number of colonies, theoretically arising from individual conidia, were selected for testing. No nicotinic acid-requiring forms were obtained. The delayed enrichment procedure, described by Lederberg and Lederberg,[15] was also tested. Plates of basal agar (casein vitamin-free agar) were serially streaked. On plates where the colonies were well dispersed, their locations were marked on the backs of the plates. After a few days, sterile penicillin cups were placed in the centers of each plate and a dilute solution of nicotinic acid was added. The plates were again examined after several days; however, no new colonies appeared.

Similar experiments were carried out using the *T. mentagrophytes* culture which had shown some stimulation by nicotinic acid (No. 17 in this series). In this case, it was possible to select colonies which showed greater stimulation than others. No colonies with a complete requirement for this vitamin were found.

## Summary

1) It is suggested that *Trichophyton equinum* (Matruchot and Dassonville) Gedoelst, 1902, be considered as a valid spe-

cies distinct from *Trichophyton mentagrophytes*.

2) *Trichophyton equinum* is a common cause of ringworm of the horse in the United States, Europe, and South America.

3) *Trichophyton equinum* has a characteristic colonial form and pigmentation and has a complete nutritional requirement for nicotinic acid. The manner of the invasion of the infected hair may be described as ectothrix with medium to large spores (3.5 to 8.0 $\mu$). These characteristics distinguish it from all other dermatophyte species.

4) Clinically, the disease produced in horses by *T. equinum* is similar to that produced by other dermatophyte species.

5) Five different dermatophyte species have been found associated with ringworm infections of the horse. In order of frequency these are: (1) *T. equinum*, (2) *Microsporum canis*, (3) *T. mentagrophytes* var. *granular*, (4) *Trichophyton verrucosum*, and (5) *Microsporum gypseum*.

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# Persistence of Viremia and Immunity in African Swine Fever

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AFRICAN SWINE FEVER (ASF) is maintained in nature in wart hogs (Phacochoerus sp.) and bush pigs (Potomochoerus sp.).[1-5] Infected wild swine are inapparent carriers and appear to suffer no ill-effects from the virus. However, in domestic swine, ASF virus produces a highly contagious, peracute infection which clinically resembles acute hog cholera but immunologically is distinct from it.

Montgomery[1] reported a mortality of 98.9 per cent in 1,276 domestic swine involved in ASF in Kenya from 1910 to 1912. Survivors of natural infection were rare, and attempts to immunize against ASF have for the most part failed. Thus, few survivors of ASF in domestic swine have been available for immunological and other studies.

Between the initiation of this project in July, 1954, and February, 1956, there were 13 survivors of 217 domestic pigs exposed to ASF virus. The term "survivor" is used, with reservations, to describe those pigs that lived for significantly longer periods than usual after exposure to ASF virus. The period of survival varied from 24 to 456 days. The average interval from exposure until death of those which developed typical ASF was 17.4 days for 38 pigs which were exposed by contact and eight and one-half days for 82 pigs exposed by inoculation.

Since all 13 of the so-called survivors eventually died of either chronic or acute ASF, no case of survival was based on true immunity. A persisting viremia was demonstrated in 8 of the 13 survivors. Two did not show a persisting viremia when tested and 3 were not tested. Thus, 8 of the so-called survivors actually had chronic cases of ASF and were also carriers.

When serum from the first survivor in this series protected a second pig against a simultaneous virus challenge, the possibility of developing a successful antiserum against ASF virus was considered. However, later experiences with this method of immunization were discouraging.

This paper is essentially a report on the 8 surviving swine which showed persisting viremia. The theory is advanced that the resistance of surviving domestic swine to subsequent ASF exposure may depend on a persisting infection rather than on the persistence of circulating antibodies. In addition, the potential danger of domestic swine as carriers of ASF is re-emphasized.

## LITERATURE CITED

Walker[2] was unable to show that the blood of 3 ASF-recovered domestic pigs was infectious, but Steyn[3] demonstrated ASF viremia in 2 pigs two months following a severe reaction induced by inoculation of infectious wart hog blood. Steyn mentioned that one South African outbreak of ASF probably resulted from feeding blood from abattoirs where ASF carriers had been slaughtered. DeKock *et al.*[4] recognized that domestic swine which survived an epizootic of ASF might remain carriers. They stated that in one instance susceptible pigs died of ASF when placed with survivors of an attack which had occurred seven months previously. These workers also demonstrated ASF virus in the blood of 6 of 10 pigs which survived natural infection. Two of these survivors were still carriers when slaughtered ten months later. However, 2 pigs which survived experiments and were subsequently resistant to ASF virus inoculations did not show persisting viremia.

Walker[2] stated that killed ASF antigen conferred no immunity, and DeKock *et al.*[4] had no success with crystal-violet vaccine. Montgomery[1] and Steyn[3] failed to immunize swine against ASF by means of virus and antiserum, but Walker[2] produced a hyperimmune serum which protected pigs against death from simultaneous injections or contact exposure to virus of the same origin with which the serum donors had been hyperimmunized. He found that the immunity conferred by the serum simultaneous method still existed at 192 days, but not at 283 days.

## MATERIALS AND METHODS

Large White pigs, weighing 40 to 80 lb. when first exposed, were used. They were from stock imported from England but were raised in the ASF-free area of Kenya.

To test for ASF viremia, from 1.0 to 10.0 ml.

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of blood taken from a suspected carrier was inoculated into a susceptible pig. Blood was taken from the anterior vena cava of the suspect and placed in a 1-oz. bottle containing anticoagulant. Modified Alsever's solution, 2 per cent sodium citrate, and heparin were used as the anticoagulants at various times. The Alsever's solution and 2 per cent sodium citrate were used in equal parts with suspected blood. A solution containing 10 mg. per milliliter of sodium salt of heparin was used, and 0.2 ml. of this solution was mixed with each 10.0 ml. of blood. Sodium citrate appeared to have some virucidal effect, so heparin was selected as the anticoagulant of choice for this work. The susceptible test pigs were inoculated within 15 minutes after the blood samples were taken. The subinoculated pigs were held in isolation stalls and their temperatures taken morning and night. The viremia test was called positive if the subinoculated pigs showed typical clinical signs of ASF and typical lesions at necropsy. The test was called negative if the subinoculated pig remained normal and was later susceptible when challenged with ASF virus.

Serum was obtained from blood samples taken from the anterior vena cava or from the tail of surviving pigs. All serums were inactivated by heating for 30 minutes at 55 C. The presence of antibodies was determined by serum-protection tests in pigs against simultaneous virus inoculation, or contact exposure, or both. One attempt was made to detect antibodies by an *in vitro* serum-neutralization test.

Crystal-violet ASF vaccine was prepared according to the production method described by Cole and Henley[4] for hog cholera. An aluminum hydroxide-adsorbed vaccine was prepared by mixing equal parts of ASF blood and aluminum hydroxide gel (aluminum oxide content 1.5%). Formalin was added to a concentration of 0.5 per cent, and the vaccine was incubated for 72 hours at 37 C. Four 50-lb. pigs were each given doses of 5.0, 8.0, and 10.0 ml. of the inactivated vaccines (2 were given crystal-violet and 2 the aluminum hydroxide) at 14-day intervals. Twelve days after the final dose, the pigs were challenged with 1.0 ml. of the same virulent virus blood from which the vaccines were made.

With one exception, the Hinde strain of ASF virus, isolated from an animal with a field infection in Kenya in August, 1954,[5] was used throughout the experiments. Defibrinated blood was used as the stock challenge virus and had an $l.d._{50}$ titer of $10^{-6}$, using 2 pigs for each tenfold dilution. The inoculation of 1.0 ml. of this virus alone never failed to kill control pigs, after an average interval of eight and a half days. This stock challenge virus was stored at −20 C. In the single exception, the South African Spencer strain of ASF virus, obtained from the Onderstepoort laboratory, was used as the challenge virus.

## Results

A summary of the viremia and antibody tests on the 8 surviving pigs which showed a persisting viremia is given (table 1). A

**TABLE 1—Summary of Viremia and Antibody Tests on 8 Domestic Swine Survivors of African Swine Fever**

| Pig No. | Days survived PE* | Viremia tests | | Antibody tests | |
|---|---|---|---|---|---|
| | | Days PE | Results | Days PE | Results |
| 2093 | 456 | 27 | Positive | 27 | Positive |
| | | 49 | Positive | 49 | Negative |
| | | 85 | Positive | 141 | Negative |
| | | 128 | Positive | ..... | ..... |
| | | 162 | Negative† | ..... | ..... |
| | | 172 | Positive | ..... | ..... |
| | | 352 | Negative | ..... | ..... |
| | | 369 | Negative | ..... | ..... |
| | | 440 | Positive | ..... | ..... |
| 2117 | 107 | 15 | Positive | 15 | Positive |
| | | 33 | Positive | 33 | Positive |
| | | 94 | Positive | 99 | Negative |
| 2122 | 86 | 78 | Positive | Not made | ..... |
| 2127 | 362 | 60 | Positive | 73 | Negative |
| | | 94 | Negative† | ..... | ..... |
| | | 104 | Positive | ..... | ..... |
| | | 284 | Positive | ..... | ..... |
| 2144 | 169 | 50 | Negative† | 138 | Negative |
| | | 138 | Positive | ..... | ..... |
| 2142 | 229 | 50 | Positive | 138 | Negative |
| | | 138 | Positive | 173 | Negative |
| | | ..... | ..... | 207 | Negative |
| 2249 | 90 | 41 | Positive | 41 | Negative |
| 2247 | 119 | 41 | Positive | 41 | Negative |
| | | 105 | Positive | ..... | ..... |

* PE = postexposure.

† Blood, placed in equal parts of 2 per cent sodium citrate, which may have been virucidal.

brief history of each of these 8 carriers follows:

1) Pig 2093, the first and longest survivor in this series of experiments, lived 456 days after it was first exposed by contact with 3 pigs which died of acute ASF. Pig 2093 showed a febrile peak of 107.2 F. on postexposure (PE) day 13 and was so sick it could no longer stand unaided. However, a week later, the pig was eating and the temperature was normal. It continued in poor condition for two months and developed pronounced swellings of the hocks and under the mandible but it gradually improved and appeared normal until a few weeks before death. During the 15 months of its survival, 23 pigs died of acute ASF in the same stall, thus providing 2093 with almost constant exposure to ASF virus. It showed periodic febrile reactions over 104.0 F., but four of the six positive

viremia tests (table 1) were made when no febrile reaction was present. It was negative when tested for viremia on PE days 352 and 369 but viremia was again shown to be present on day 440. On PE day 27, blood was obtained from 2093 for serum. Antibodies were demonstrated in this serum by a protection test made on pig 2117. Since a viremia test made on day 27 also was positive, it would appear that, at this time, there was coexistence of virus and protective antibodies in the blood of 2093. Thirty milliliters of serum taken from 2093 on PE day 49 and 70.0 ml. taken on day 141 failed to protect susceptible pigs against either simultaneous virus inoculation or contact exposure to ASF Hinde virus.

2) Pig 2117 sickened but survived for 107 days after a simultaneous inoculation of 45.0 ml. of serum from pig 2093 and 1.0 ml. of ASF Hinde virus. A control pig receiving the virus alone died seven days after inoculation. Following exposure, pig 2117 had a febrile reaction reaching a peak of 107.2 F. on day 5. Serum taken from pig 2117 on PE days 15 and 33 contained antibodies which were demonstrated by protection tests made on pigs 2122 and 2127. Sixty milliliters of serum taken from 2117 on PE day 99 failed to protect 2 pigs against simultaneous inoculations of 1.0 ml. of ASF Hinde virus. As in pig 2093, 2117 appeared to show coexistence of viremia and protective antibodies on PE days 15 and 33.

3) Pig 2122, exposed to ASF-infected pigs immediately following the inoculation of 20.0 ml. of serum from pig 2117, sickened but survived for 86 days. Its febrile reaction started on PE day 11 and reached a peak of 107.0 F. on day 14. A control pig similarly exposed died on day 17.

4) Pig 2127 sickened but survived 362 days following the simultaneous inoculation of 45.0 ml. of serum from pig 2117 and 1.0 ml. of ASF Hinde virus. The febrile reaction reached a peak of 105.2 F. on day 7. A control pig given the virus alone died on PE day 9. Fifty-five milliliters of serum taken from pig 2127 on PE day 73 failed to protect another pig against a simultaneous challenge of 1.0 ml. of ASF Hinde virus.

5) Pig 2144 survived for 169 days after being placed in contact with the 4 previous surviving carrier pigs. Another pig similarly exposed died on day 18. Pig 2144 also survived contact with pigs dying of acute ASF on PE days 17, 30, 68, 72, and 115. In addition, it survived the inoculation of 5.0 ml. of virulent ASF Hinde virus blood on PE day 60. It was the first of 2 pigs that became carriers without first showing signs of ASF. Sixty milliliters of serum taken from pig 2144 on PE day 138 failed to protect a pig against a simultaneous challenge of ASF Hinde virus.

6) Pig 2142, which was given the same contact exposure as 2144, survived for 229 days. It also survived inoculations of ASF Hinde virus as follows: 2.0 ml. on PE day 142, 60.0 ml. on day 158, and 500.0 ml. on day 190. Sixty to seventy-five milliliters of serum taken from 2142 on PE days 138, 173, and 207 failed to protect susceptible pigs against simultaneous virus inoculations or contact exposure. One milliliter of undiluted serum taken from pig 2142 on day 173 failed to neutralize 100 $l.d._{50}$ of Hinde virus after two hours at 20 C. *in vitro*. Pig 2142 was the second of 2 pigs which became carriers without first showing signs of ASF.

7) Pig 2249 became ill but survived for 90 days after contact with 4 pigs which died of acute ASF. The febrile reaction reached a peak of 104.6 F. on PE day 16. Two other pigs given the same exposure died on day 16.

8) Pig 2247 also sickened after the same contact exposure as 2249 but survived for 119 days. A febrile reaction commenced on day 20 and reached a peak of 105.0 F. on day 22. It also survived for 69 days after an inoculation of 2.0 ml. of the South African Spencer strain of virulent ASF virus on PE day 50. A control pig challenged with the South African virus died on day 9. Fifty milliliters of pooled serums from pigs 2249 and 2247 taken on PE day 40 failed to protect a pig against simultaneous inoculation of 1.0 ml. of ASF Hinde virus.

In addition to the 8 carrier pigs, there were 2 survivors in which viremia could not be demonstrated. A history of 1 of these pigs follows.

Pig 2129 showed no illness after contact exposure to 6 pigs which died of acute ASF but developed a temperature of 104.2 F. on PE day 6 and 104.4 F. on day 7. A viremia check on day 36 was negative. On day 48, pig 2129 was again exposed by contact with a pig that died of acute ASF.

As a result of this second exposure, pig 2129 sickened, showing a febrile peak of 107.4 F. on day 10, and died on day 15.

*Results with Inactivated African Swine Fever Vaccines.*—When 4 pigs which were vaccinated with the crystal-violet and aluminum hydroxide-adsorbed vaccines were challenged with ASF virus, they sickened typically and died. These negative results with inactivated ASF vaccines confirmed the findings of Walker [3] and DeKock *et al.*[4]

*Clinical Observations and Postmortem Findings in African Swine Fever Carrier Pigs.*—As previously stated, all but 2 of the surviving carrier pigs showed febrile reactions at irregular intervals throughout the period of survival. Soft edematous swellings developed over the leg joints in most animals and under the mandible in 1 animal. Some of the pigs showed stiffness when forced to move. The articular surfaces appeared normal at necropsy. For two to three weeks prior to death, coughing and signs of pneumonia were present.

Death in the carriers appeared always to be associated with chronic lesions initiated by the virus and perhaps intensified by the persisting infection. The most characteristic finding at necropsy was a pronounced chronic pericarditis. Adhesions of the pericardium to the heart and to the surrounding lung tissues were invariably present. A grayish white, gritty, caseous exudate was sometimes found in the pericardial sac. No bacterial growth occurred when this material was cultured on agar slants and in serum broth. The epicardium was blanched. DeKock *et al.*[4] described extensive pericarditis as being a rather constant feature of ASF carriers at necropsy.

In 2 animals, the lungs were almost completely consolidated. Extensive adhesions of the lungs to the thoracic wall occurred. Several hundred milliliters of amber fluid was often present in the thoracic cavity. Up to 2 liters of this fluid was found in the abdominal cavity. Various degrees of gastritis and enteritis were present. In pig 2093, the spleen was about twice the normal size and very soft.

## Discussion

Although protective antibodies were apparently present in the serums of pigs 2093 and 2117 shortly after they were first exposed, no such antibodies were demonstrated later by the methods used. Antibodies were never demonstrated in the serums of the other 5 pigs examined. Thus, the antibody response, if any, seemed to be of short duration. In contrast, viremia became established and persisted. Accordingly, ASF seems to fit into Francis'[7] group 1 of virus diseases, *i.e.*, virus diseases in which virus infection persists but immunity does not. Concerning this group, Francis states, "The failure to respond to inoculation of the same agent with a chain of reactions similar to that exhibited by the uninfected host is commonly called infection immunity. It is scarcely immunity but a stage of tolerance to amounts of infectious agent which appear insignificant in terms of the amount available in the tissues of the infected host." Francis' classification provides a further point of differentiation of ASF from typical hog cholera which fits into his group 2—virus diseases in which immunity persists and evidence indicates that virus does not.

It appears possible, from the limited work reported here, that a persisting ASF infection must be established in domestic pigs before resistance develops to re-exposure with ASF virus. Thus, instead of the sterile immunity often inducible against virus diseases, there may be something more nearly approaching the premunition which occurs in some of the protozoal blood diseases such as piroplasmosis and anaplasmosis of cattle. Premunition depends on the persistence of a carrier infection which renders the host resistant to reinfection. In these two protozoal diseases, there appears to be no immunity in the absence of carrier infection.

African swine fever, by exhibiting persisting infection in the absence of demonstrable protective antibodies, is not unique among virus diseases. Francis places the agents of the psittacosis-lymphogranuloma venereum group in his group 1 category. Equine infectious anemia [8] and serum hepatitis of man [9] are additional examples and further resemble ASF in that viremia tests may prove the blood of the carrier fully virulent for a susceptible host.

Pig 2093, by showing a remission and reappearance of viremia, presents an interesting case for conjecture. Three possible explanations follow:

1) Pig 2093 may have become completely free of ASF virus and was rein-

fected from continual contact exposure to which it was subjected. If this were the case, the theory of infection immunity or premunition would be strengthened.

2) During the period of remission, the virus remained present in a tissue or tissues and was later released from the focal areas into the blood stream.

3) The initial production of protective antibodies may have continued even though they were undetected by the methods used. If true, it may be possible that at times the antibody production caused the viremia to be reduced to such a high dilution factor that virus was not present in the small amount of blood injected into the susceptible pig.

The failure of pig 2129 to show illness after the first contact exposure may have resulted from some innate resistance in the pig. Although it survived the first exposure, viremia did not become established. When it was re-exposed by contact 48 days later, 2129 sickened typically and died of acute ASF. The experience with this pig further supports the theory of infection immunity in ASF.

Walker [3] suggested that in ASF there exist differences of virus strains, or of virulence, or of both. He used this reasoning in an attempt to explain the breakdown in immunity in pigs later challenged with virus from a source other than that of the virus used in his simultaneous serum-virus immunization studies. However, in the case of carrier pig 2247, no reaction followed a challenge with the South African virus, even though the original exposure was to the Kenya Hinde virus. Recently, workers [10] at Onderstepoort, South Africa, reported that a few pigs which survived infection with egg-propagated ASF virus were immune to the homologous virus but not to a strain of ASF virus obtained from the Belgian Congo. The question as to whether different immunological types or strains of ASF exist will not be entirely resolved until cross-immunity tests can more readily be conducted or until a serological method of identifying the virus is developed.

It is perhaps unwise to draw so many inferences from the relatively few observations reported here. It should be remembered that these observations were made almost entirely on a single strain of ASF virus and that other strains may not necessarily behave similarly. Since survivors to ASF are rare, it may be justifiable to report this work in an attempt to further elucidate this complex problem.

According to these observations little can be added to Steyn's statement [2]: "The fact that pigs which have recovered from an attack of virus disease [ASF] harbour the virus precludes the use of vaccine which contains live virus, as the immunization of pigs by this means will create 'carriers.' In the light of our present knowledge the only way of combating virus disease of pigs [ASF] is to slaughter all affected and in-contact animals and to quarantine affected localities."

One additional warning should be made. The experience with pigs 2144 and 2142 shows that subclinical ASF infections can occur in domestic pigs. It would appear that this could also occur in nature, thereby establishing ASF as an enzootic disease in domestic pigs.

Whether a suitable live virus vaccine against ASF can be developed remains to be seen. According to the theory presented here, such a vaccine would have to induce a persisting infection in order to give protection. If true, the problem of developing a satisfactory attenuated live virus vaccine would be extremely complex.

## SUMMARY

1) Eight domestic pigs, which survived for periods of 86 to 456 days after African swine fever (ASF) exposures, showed a persistence of viremia which was demonstrated by subinoculating susceptible pigs.

2) Two of the 8 pigs showed a co-existence of viremia and circulating antibodies. Protective antibodies were demonstrated in the serums of these 2 pigs 27 and 33 days, respectively, after the initial exposure, but could not be demonstrated thereafter although the viremia persisted.

3) Two of these domestic pigs showed ASF viremia without showing clinical reaction to the original exposure.

4) Domestic pigs, as well as wart hogs and bush pigs, may become carriers and potential spreaders of ASF.

5) Killed antigen vaccines failed to induce immunity to ASF.

6) Resistance to reinfection with ASF virus may depend on a persisting infection rather than on the persistence of circulating antibodies.

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# A Disease of Swine and Cattle Caused by Eating Moldy Corn
## II. Experimental Production with Pure Cultures of Molds

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In an earlier paper, Sippel, Burnside, and Atwood[8] reviewed the literature and described the occurrence of poisoning of swine and cattle, caused by eating moldy corn, which occurred in the fall of 1952. The results of the initial experimental work on this problem were presented. Additional articles have appeared dealing with toxic molds. Hieashi[5] reported a toxic Penicillium isolated from stored rice. Two toxic substances were reported to be present. Methanol extracts of the moldy rice and mycelium from liquid cultures were equally toxic for rats. Oral administration of the moldy rice *per se* was also intoxicating.

### Review of Literature

Gajdusek[4] and Mayer[6,7] called attention to mycotoxicoses reported in Russia for a number of years. Molds belonging to the genus Fusarium, particularly *Fusarium sporotrichoides*, were reported as the most common cause. The Russian workers found that the toxic substances were produced in grain that was harvested after remaining beneath the snow throughout the winter. Grain harvested before the spring thaw was reported to be nontoxic. The toxic substance was reported to be stable up to 200 C. and grain proved toxic after a four-year storage period. Where the disease was reported, 5 to 40 per cent of the grain samples cultured contained this mold, while in other areas, the fungus was found in only 2 to 8 per cent of the grain cultured. The soil was reported to be the reservoir for this fungus.

Various Russian workers[4] reported a correlation between the toxicity of such grain and a local reaction observed when ether extracts of the grain were applied, topically, to shaved areas on the backs of rabbits. They also mentioned moldy bread poisoning (*Fusarium graminearum*) in man as well as other toxicoses due to *Stachybotrys atra*. These latter two seemed to be of less importance.

In this country, Carll *et al.*[2] have isolated three species of Aspergillus from a food concentrate which produced some signs of bovine hyperkeratosis. These workers showed that an ether extract of one of these molds, *Aspergillus chevalieri*, was capable of producing hyperemia when applied topically to the skin of a rabbit or calf. They further showed that 23.0 and 16.8 lb. of sterile bread, on which a pure culture of this mold was grown, were toxic to 2 calves in five and four days, respectively.

A similar study with *Aspergillus tomarii* and *Aspergillus flavus-oryzae* gave negative results.

Forgacs and co-workers[3] reported the isolation of a toxic strain of *Aspergillus clavatus* from a pelleted feed, which produced hyperkeratosis in cattle and was reported to be free from highly chlorinated naphthalenes. Ether extracts of various substrates on which this mold was grown showed dermal toxicity in rabbits, antibacterial activity against a strain of *Bacillus subtilis*, and were toxic to mice when administered by the subcutaneous, intraperitoneal, and oral routes. *Aspergillus clavatus*-inoculated corn produced both acute and chronic intoxication when force fed to calves. Deaths were produced by feeding 4.5 lb. daily for six days and 11.5 lb. daily for 13 days. Chronic intoxication was produced by feeding 1 lb. daily for at least 85 days. The weights of the animals were not given. Calves that died suddenly from *A. clavatus* and *A. chevalieri* toxicoses showed similar signs. The techniques for chemical extraction and testing of the extract for antibacterial activity and toxicity were discussed.

The moldy corn poisoning reported in 1952[8] involved about 1,000 swine. From August to November, 1953, moldy corn poisoning involving 533 swine was again diagnosed at this laboratory. In both years, at the time the animals were presented to the laboratory, there was a mortality of 22 per cent among the diseased swine in the area. Only a small fraction of the poisoned animals in the region were referred to the laboratory.

### Materials and Methods

Samples of corn which was suspected of being toxic were collected from various areas where

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toxicoses had occurred in the fall of 1952. These samples were macerated separately by mortar and pestle under aseptic conditions. Each sample was serially diluted in sterile distilled water, plated on Czapek's solution agar and Mycophil agar, and incubated at 24 C. Thirteen cultures of fungi were isolated from these samples by this procedure.

Under our laboratory conditions, poisoning in swine could be produced by feeding, via stomach tube, moldy corn gathered from the fields where the disease had been reported. It then became necessary to screen the 13 suspected toxic fungi for toxicity to swine. This paper reports the results of feeding swine pure cultures of the molds after they had been grown on a substrate of sterile, whole grain corn.

A variety of early, soft, corn was selected for the substrate since it was the type of corn most often consumed by the affected swine.

All stock cultures were maintained on Czapek's solution agar except a species of Penicillium which was maintained on Mycophil agar.*

Inoculum for the corn was prepared by adding a loopful of spores from a well-sporulated slant, or 1 ml. of a suspension of the spores to 500-ml. Erlenmeyer flasks containing 250 ml. of Czapek's solution broth. Inoculum from the Penicillium was prepared in the same manner except that the sucrose content of the broth was increased to 20 per cent. The cultures were incubated for two weeks at 26 C.

The corn was soaked in tap water overnight to soften the kernels and permit better heat penetration during autoclaving. The following morning, it was drained and five, 1-gal. carboys were filled about one fourth full or, when possible, 2-inch layers were placed in 4-liter Fernbach flasks. These were autoclaved for one hour at 15 lb. pressure.

When cool, the corn in each container was inoculated with about 125 ml. of the inoculum and incubated at 26 C. for two to three weeks. Each day, the containers were shaken vigorously to prevent clumping of the corn.

When the incubation period was completed, the corn was dried in a large drying oven at 60 to 70 C. A drying period of 26 hours was usually sufficient. As the final step, the corn was ground sufficiently fine so that, in aqueous suspension, it could pass readily through a stomach tube.

One volume of the finely ground moldy corn (0.5 to 1.0 lb.) was added to 3 to 4 volumes of tap water to make a suitable suspension. This was administered to the experimental pigs by pouring it through a funnel attached to a stomach tube. The control pigs were fed uninoculated corn in the same manner. After the routine was thoroughly established, the force feeding of control pigs was omitted except for periodic checks. The feeding process was continued for a maximum of ten to 13 days. Blood samples were taken from all pigs, at least three times during the trial, and analyzed for vitamin A, hematocrit, and hemoglobin values, and the leukocyte and erythrocyte counts and icterus index were determined.

In addition, the temperatures of all pigs were taken in the morning and at night. Complete necropsies as well as histological examinations were made on all pigs that died.

Further toxicity tests were made on the isolates from the moldy corn, using mice as the test animals. The 13 fungi were each cultured on 10-ml. Mycophil agar slants for two weeks at 24 C. When abundant growth was observed, 5 ml. of water was added to each slant and, after preliminary maceration with a stirring rod, the fungus-agar suspension was transferred to a Tenbroeck tissue grinder and macerated until the suspension could pass readily through an 18-gauge needle. Mice (10 per group), weighing approximately 23 Gm. each, were then force fed with a syringe and blunted needle. All mice in a group were given 0.5 ml. of the appropriate fungal suspension daily for six days or until death occurred. The control mice were given 0.5 ml. of sterile Mycophil agar prepared in the same way but on which no fungi had been cultured.

Ground Penicillium-corn substrate was sent to Dr. H. R. Doll of the University of Kentucky to be fed to horses. Quantities varying from 500 Gm. to 4 kg. were mixed with water, allowed to settle, and the supernatant fluid given to 4 horses. The total erythrocyte and leukocyte counts, hemoglobin values, and differential leukocyte counts of these horses were determined during the feeding period.

In more recent work, additional methods of screening molds for toxicity have been used. Mold isolates have been grown in broth for ten days, passed through a Swinny filter (Becton-Dickerson Co.), and the air sacs of 10-day embryonating eggs inoculated with 0.2 ml. of the filtrate.* The eggs were observed for embryo viability for three days. A 10 per cent dilution of the filtrate in broth was used to test for activity against *B. subtilis*. The filter pad was placed on a blood agar plate in such a way that one side was in contact with a streak of *Escherichia coli* while the other side touched a streak of Micrococcus sp. Molds found to be exhibiting activity by any of the above tests were tested further by intraperitoneal inoculation of culture filtrate into guinea pigs or young chicks, or by feeding cultures to the animal species originally involved.

## RESULTS

Taxonomic studies† of the 13 mold isolates showed that nine were representative of the species *Aspergillus flavus* Link, three were representative of the species *Fusarium moniliforme*, and the remaining one was *Penicillium rubrum* Stoll. Feeding

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* We are indebted to Dr. R. G. Gentry and associates of Pennsylvania State University for this technique.

† The authors thank Dr. Kenneth B. Raper, Department of Bacteriology, University of Wisconsin, Madison, who identified these cultures.

pure cultures of mold to swine produced negative results, as measured by the criteria used, except in two instances.

The first culture to prove toxic was a strain of *A. flavus* Link. When the first 2 pigs (38 and 50 lb.) had succumbed to the mold-corn substrate, 2 more pigs (40.0 and 47.5 lb.) were fed the same substrate. The material was toxic to all 4 pigs (trials I and II, table 1). A second batch of sterile corn was inoculated and the experiment repeated. In trial III (table 1), 2 of the 4 pigs (57.0 and 44.5 lb.) served as controls. With one exception, the Aspergillus-corn substrate produced death in the 6 experimental animals after four to five days.

The second culture that proved toxic was *P. rubrum* Stoll. The toxicity of the initial batch of the Penicillium-corn substrate (trial I) was confirmed in trial II. Due to the extreme toxicity of this mold, a series of trials was undertaken to approximate the minimum amount of the moldy substrate that would prove toxic to swine.

Five mixtures of corn were prepared that contained 1.25, 2.5, 5.0, 10.0, and 20.0 per cent of the Penicillium-corn substrate. Each mixture was fed to 2 pigs. These results are summarized (trials III–VII, table 2).

To further test the toxicity of *P. rubrum* Stoll, another batch of sterile corn was inoculated. This was toxic to a male goat (trial VIII) and was still toxic to a pig (trial IX) after a storage period of 61 days in a cool, dry place.

With the exception of vitamin A levels, the measurements made on the blood samples were in the normal range and no definite trends were established. Three of the cultures represented by the species *A. flavus* Link, that did not produce signs of poisoning, appeared to have a detrimental effect on plasma vitamin A levels. During the test period, these three cultures decreased the initial plasma vitamin A levels from averages of 32, 21, and 19 to 9, 6, and 4 γ/100 ml. of plasma, respectively. The toxic strains of *A. flavus* Link and the *P. rubrum* Stoll produced death so quickly that plasma vitamin A levels were not determined.

When sublethal amounts of the Penicillium-corn substrate were fed, *i.e.*, the 1.25 to 10.0 per cent mixtures, plasma vitamin A levels tended to increase. In these trials, the average initial plasma vitamin A levels of the 2 pigs given the 1.25 to 5.0 per cent mixtures of Penicillium-corn substrate increased from 35 to 46 γ of vitamin A per 100 ml. of plasma. The average vitamin A level of the 2 pigs given the 10 per cent mixture also increased, from 19 to 46 γ of vitamin A per 100 ml. of plasma.

At various times during the entire experiment, an increase in temperature of 1 to 3 degrees (F.) occurred in some of the animals. These increases were not characteristic of any particular mold substrate nor was a temperature pattern formed.

The experiment with mice confirmed the results of the experiment with swine in that all mice remained normal except the groups which were given the two cultures that proved toxic to swine, *i.e.*, *P. rubrum* Stoll and the toxic strain of *A. flavus* Link.

The mice in these two groups became depressed at the end of 24 hours and some died during the second day. By the end of

**TABLE 1—Results of Feeding Aspergillus Flavus-Corn Substrate to Swine**

| Trial No. | Pig wt. (lb.) | Daily dose (lb.) | No. of days dosed | Total dose (lb.) | Time of death | Day of onset and signs of poisoning |
|---|---|---|---|---|---|---|
| I *A. Flavus*-corn substrate | 38.0 | 1.75 | 5.0 | 8.75 | 5th day | 5th—bleeding from anus; great discomfort, continual picking up feet; weakness. |
| | 50.0 | 1.75 | 9.5 | 16.62 | 10th day Killed | 5th—bleeding from anus. 6th—icterus; moribund condition. |
| II *A. Flavus*-corn substrate | 40.0 | 1.75 | 4.0 | 7.00 | 4th day | 3rd—icterus. |
| | 47.5 | 1.75 | 3.0 | 5.25 | 3½ days | 3rd—icterus. |
| III *A. Flavus*-corn substrate | 62.0 | 1.75 | 4.0 | 7.00 | 5th day Killed | 4th—moribund condition. |
| | 68.5 | 1.75 | 4.0 | 7.00 | 5th day | 4th—a.m., bleeding from anus. 5th—moribund condition. |
| IV Sound corn | 57.0 | 1.75 | 4.0 | 7.00 | ..... | Normal. |
| | 44.5 | 1.75 | 4.0 | 7.00 | ..... | Normal. |

the sixth day, all of the mice in each lot were dead. The mice that remained alive on the third and fourth day, in addition to being depressed, showed atony of the muscles, debilitation, and dehydration.

The 4 horses given supernatant fluid from water suspensions of the ground Penicillium-corn substrate died in 34 hours to 11 days, depending on the dose administered (table 3). The total dose varied between 3 and 5 kg. of Penicillium-corn substrate from which the supernatant fluid was taken. The erythrocyte and leukocyte counts, hemoglobin values, and differential leukocyte counts remained normal during the trial.

Only the molds found toxic in feeding trials proved toxic in the embryonating egg-inoculation or bacterial growth-inhibition tests. *Penicillium rubrum* Stoll killed the chicken embryos and inhibited growth of the Micrococcus. The toxic strain of *A. flavus* prevented the growth of *B. subtilis* in broth.

### GROSS NECROPSY LESIONS

*Swine: Aspergillus Flavus-Corn Substrate.*—The lesions in these pigs were similar to those observed in field cases. The entire carcass was icteric and there was usually a yellow-orange fluid in the peritoneal cavity. Subcutaneous hemorrhages, varying from 2 to 7 cm. in diameter, were usually found in the inguinal and axillary spaces. The lymph nodes were swollen, moderately edematous, and congested. In some nodes, the congestion was peripheral. The liver changes varied considerably. Some livers were yellow, more or less cirrhotic, and shrunken. Others were dark, swollen, and congested, with irregular hemorrhagic areas, and were normal in consistency or contained areas of cirrhosis. Dilated capillaries were seen on

**TABLE 2—Results of Feeding Swine and Goats with Penicillium Rubrum Stoll-Corn Substrate**

| Trial (No.) | Wt. of pig (lb.) | Daily dose (oz.) a.m. | Daily dose (oz.) p.m. | No. of days dosed | Total dose (oz.) *P. rubrum*-corn substrate | Time of death | Signs of poisoning |
|---|---|---|---|---|---|---|---|
| I—*P. rubrum*-corn substrate | 30.0 | 8.0 | 8.0 | 1.0 | 16.0 | 24 hr. | Both pigs lethargic throughout day, frothing at mouth, braced against wall, collapsed if made to move. |
| | 51.0 | 8.0 | 8.0 | 1.0 | 16.0 | Less than 18 hr. | |
| II—*P. rubrum*-corn substrate | 55.0 | 8.0 | 0 | 1.0 | 8.0 | Less than 18 hr. | 17 minutes after dosing, both pigs were salivating, frothing, and chomping teeth; the second hour, heads pressed against pen, moved in a hesitant and belligerent manner; sixth hour, condition same as second hour; second day, remaining pig icteric; erythema, jaw to anus on ventral surface prior to and after death. |
| | 63.5 | 8.0<br>4.0 | 0<br>0 | 2.0 | 12.0 | Less than 36 hr. | |
| III—Mixture of corn containing 1.25% *P. rubrum*-corn substrate | 36.0 | 12.0 | 12.0 | 5.0 | 1.5 | ..... | Normal. |
| | 41.0 | 12.0 | 12.0 | 5.0 | 1.5 | ..... | |
| IV—Mixture of corn containing 2.5% *P. rubrum*-corn substrate | 50.0 | 12.0 | 12.0 | 8.5 | 5.1 | ..... | Normal. |
| | 58.0 | 12.0 | 12.0 | 8.5 | 5.1 | ..... | |
| V—Mixture of corn containing 5.0% *P. rubrum*-corn substrate | 42.0 | 12.6 | 12.6 | 3.5 | 4.2 | ..... | Normal. |
| | 54.0 | 12.6 | 12.6 | 3.5 | 4.2 | ..... | |
| VI—Mixture of corn containing 10% *P. rubrum*-corn substrate | 38.0 | 12.0 | 12.0 | 12.5 | 30.0 | ..... | Normal. |
| | 47.0 | 12.0 | 12.0 | 12.5 | 30.0 | ..... | |
| VII—Mixture of corn containing 20% *P. rubrum*-corn substrate | 54.0 | 13.0 | 13.0 | 1.5 | 7.8 | Less than 32 hr. | Both pigs showed depression, and a red-purple erythema from jaw to anus on ventral surface prior to and after death. |
| | 59.0 | 13.0 | 13.0 | 1.5 | 7.8 | Less than 32 hr. | |
| VIII—*P. rubrum*-corn substrate | Male goat | 12.0 | 12.0 | 1.0 | 24.0 | Less than 18 hr. | Depression. |
| IX—*P. rubrum*-corn substrate stored for 61 days in cool, dry place | 46.5 | 8.0 | 8.0 | 1.0 | 16.0 | Less than 18 hr. | Showed depression, and a red-purple erythema from jaw to anus on ventral surface prior to and after death. |

both surfaces of the spleen. There were areas of congestion on the capsular surface of the kidneys of some animals. The kidneys were swollen and there were linear hemorrhages on the pyramids. The stomach was usually distended with feed and the mucosa of the pyloric and part of the fundic regions were usually hemorrhagic. In some animals there were irregular patches of hemorrhage in the jejunal and ileal portions of the small intestine and in the cecum and colon, resulting in the passage of free blood. In others, there was only a catarrhal enteritis. There were diffuse hemorrhages on the endocardium of one or both ventricles.

*Swine: Penicillium Rubrum-Corn Substrate.*—Death occurred quickly in these pigs, and lesions were confined to congestion of most tissues and an intense inflammatory reaction in the stomach. The fundic and pyloric portions of the stomach were hemorrhagic and covered with a copious mucus. Catarrhal enteritis was usually present.

*Mice: Agar-Fungal Suspension.*—Congestion was observed in the stomach, small intestine, and lungs. A general icterus and clay color of the liver and kidneys were also evident.

*Horses: Penicillium Rubrum-Corn Supernatant Fluids.*—Hemorrhages, varying in size from petechiae to ecchymoses 5 mm. in diameter, were the outstanding lesions found. These hemorrhages were most frequent in the parietal and visceral pleura and on the pericardium and surface of the adjacent large vessels. Petechiae were also seen in the peritoneum and mesentery. Hemorrhages were seen more frequently on the cecum and colon than on the small intestine and were especially marked along the cecocolic vessels. The lining of the stomach was moderately to intensely hemorrhagic, especially in the pyloric portion. The mucosa of the small intestine was catarrhal with many petechial, and a few large, hemorrhages in some animals. The wall of the cecum was slightly edematous in 1 animal.

Pale streaks were seen in the myocardium which often gave it a "herringbone" appearance. The skeletal musculature was pale to yellow or yellow-brown. Most of

**TABLE 3—Results of Feeding Horses with Penicillium Rubrum-Corn Supernatant Fluids**

| Trial (No.) | Sex and wt. (lb.) | Daily dose | No. of days dosed | Total dosage of *P. rubrum*-corn substrate | Time of death | Time of onset and signs |
|---|---|---|---|---|---|---|
| I | Male, 790 | 2 gal. supernatant fluid from 4 kg. of *P. rubrum*-corn substrate. | 1 | 4.0 kg. | 34 hr. | 24 hr., anorexia and slight depression;<br>30½ hr., depression, incoordination and clonic spasms, head pressed against wall;<br>32½ hr., rhythmic, clonic spasms at 30- to 40-second intervals;<br>34 hr., death. |
| II | Female, 820 | Supernatant fluid from 500 Gm. of *P. rubrum*-corn substrate. | 10 | 5.0 kg. | 11 days | 4th day, anorexia;<br>7th day, jaundice of sclera and weakness;<br>9th day, diarrhea;<br>10th day, depression, emaciation, appeared to have abdominal pains;<br>11th day, weakness, congested conjunctiva, death. |
| III | Female, 1,030 | 2.5 gal. of supernatant fluid from repeated washing of 1.5 kg. of *P. rubrum* corn substrate. | 3 | 4.5 kg. | 5 days | 3rd day, anorexia, icteric sclera, and congestion of conjunctiva, vomiting at irregular intervals;<br>4th day, abdominal pain. |
| IV | Male, 700 | 1.75 gal. of supernatant fluid from 1 kg. of *P. rubrum*-corn substrate | 3 | 3.0 kg. | 5 days | 3rd day, anorexia, sclera icteric, conjunctiva congested, head occasionally pushed against wall;<br>4th day, same but more severe;<br>5th day, incoordination, congestion of conjunctiva and nasal mucosa, dyspnea, icteric oral mucosa, prostration. |

the endocardium of the acutely poisoned animal was deeply hemorrhagic. The kidneys were congested and swollen, with petechiae on the cortical surface. Hemorrhages on the spleen varied from petechiae to large ecchymoses. Hemorrhages in the adrenal glands extended varying distances from the surface toward the medulla.

The liver of the horse most acutely infected was a greenish grey and was markedly congested, with petechiae scattered over it. The livers of other animals varied from yellow to brownish yellow, with petechiae under the capsule. The cut surface presented a "nutmeg" appearance. The carcasses were icteric in varying degrees in all but the acutely affected horse.

The brain and spinal cord were markedly congested and edematous. No areas of liquefaction were found in the brain.

HISTOPATHOLOGY

*Swine: Aspergillus Flavus-Corn Substrate.*—The outstanding change seen in the tissues of the experimental animals given *A. flavus* was acute hepatitis. All livers except one had extensive hemorrhages involving all but the periphery of the lobules. Normal hepatic cells were practically absent. The cells appeared as mere reticular outlines, necrotic, or showed marked fatty degeneration or advanced cloudy swelling. The nuclei of the hepatic cells were missing entirely, pyknotic and hyperchromatic, or swollen with hydropic degeneration. The hemorrhagic areas contained many lymphocytes and a few neutrophils. Kupfer's cells were prominent throughout the lobules. There were numerous pink-staining (hematoxylin and eosin) hyalin-like bodies in cytoplasm of some degenerating hepatic cells. These varied in size from mere specks to objects about six times the size of an erythrocyte. In some cells, these bodies appeared to be forming from the cytoplasm. They stained pink with Mallory's phloxine-hyalin stain. Fat stains revealed large amounts of fat in the damaged hepatic cells. The picture was that of acute, diffuse hepatic necrosis.

The epithelium of the convoluted tubules of the kidneys was moderately damaged. The nuclei of some cells were missing and the cytoplasm of the cells revealed albuminous degeneration. Microscopic lesions in other organs were not observed consistently.

*Swine: Penicillium Rubrum-Corn Substrate.*—The lesions resulting from acute poisoning with this mold were most prominent in the liver and kidney. The lesions in the liver varied widely in severity while those in the kidney were more uniform.

There was a direct correlation between the severity of the kidney lesions and the time interval from the first dose of moldy corn until death, but no such correlation existed with regard to the liver lesions.

Changes seen in the liver varied from slight swelling, nuclear degeneration, and cytoplasmic granularity of the peripheral cells to marked centrolobular necrosis. There was midzonal vacuolization of hepatic cells in other sections. Sudan IV stains of all liver sections were negative for fat. The changes in the liver were those of slight to moderate, intoxicative hepatitis.

In the kidneys the proximal convoluted tubules were the site of the most marked changes. These varied from marked cloudy swelling, in animals that died in about 12 hours, to necrosis of the cells, many of which were desquamated, in those dying in 36 hours. The tubules were dilated in some areas in a few kidneys. In the cases of longer standing, the epithelium of the distal and collecting tubules had albuminous degeneration, necrosis of a few cells, and the lumens contained many desquamated epithelial cells from higher up in the tubule.

The capillaries in the glomerular tufts and the vessels elsewhere in the organ were congested. There were collections of an amorphous material in Bowman's space in some of the glomeruli.

The lesions were those of moderate-to-severe nephrosis.

*Mice: Agar-Fungal Suspension.*—The liver showed focal necrosis and vacuolization of the cells which contained pleomorphic, nondescript bodies that stained red with Giemsa's stain. The kidneys showed dilation of the collecting tubules, amorphous tubular casts, and necrosis of the epithelium of the collecting tubules. Hemorrhages were observed in the medullary region of the kidneys.

*Horses: Penicillium Rubrum-Corn Supernatant Fluids.*—The histopathological changes were most severe in the kidneys, livers, central nervous systems, stomachs, and adrenal glands. Those in the livers and kidneys were similar to, but more severe than those seen in swine.

In horse 4, sections of the cerebellum were extensively hemorrhagic and edematous and some areas were necrotic. The molecular layer appeared to have numerous slits due to vessels that were originally dilated being shrunken by fixation. (Signs of mania shown by the horses were evidently caused by the marked meningeal congestion and hemorrhages.) No areas of liquefaction were found.

Congestion and hemorrhage in the adrenals extended from the capsule into the zona arcuata and the horse that lived 11 days had thrombosed vessels in the medulla. There was some hemorrhage and necrosis of the cells in the zona fasciculata and zona reticularis.

The stomachs had lost most of their mucosae and the submucosae were edematous and congested. The same changes were present to a lesser extent in other parts of the digestive tract.

The lesions were those of acute nephritis, hepatitis, and vascular congestion, with some hemorrhages elsewhere in the body.

## Discussion

Molds have long been suspected of being the cause of various troubles in livestock.[1] However, of 13 fungi representing various genera and species, isolated from samples of corn collected in fields where swine died, only two were toxic, even when given in massive doses. This would indicate that finding large numbers of molds in a feed or feed ingredient does not necessarily signify toxicity. Even if one of the two molds found to be toxic, or other molds known to be toxic, are recovered from a feed sample, they must be present in sufficient quantity for a given feed to prove toxic to animals.

Because of the extreme toxicity of the two molds reported here, as well as the information provided by personal communications with other research groups and unpublished data from this station, it would seem that a new emphasis should be placed on studies dealing with toxic molds.

With the possible exception of the plasma Vitamin A value and the icterus index, the blood analyses revealed no trend that would be of help in a laboratory diagnosis of an acute mold toxicosis. In our experience, mold poisoning must be diagnosed from the history, clinical signs, plasma vitamin A determinations, and gross and histological lesions. If possible, the suspected feed should be given, under controlled conditions, to animals of the species involved in field occurrences of poisoning. When time and facilities are available, the molds should be isolated and identified.

Four methods or tests by which the toxicity of molds may be determined have been described: (1) feeding cultures grown on various substrates to experimental animals; (2) dermal reactions caused by mold extracts; (3) the determination of the toxicity of culture filtrates in embryonating eggs;[1] and (4) antibacterial tests with mold culture filtrates on selected organisms.

The danger of using a fixed, arbitrarily chosen time of growth for testing growth products of mold cultures has been indicated by Waksman,[9] who has shown that the antibiotic as well as the toxic-substance level of mold cultures varies with different species and with the age of the culture. For critical study, the above observations should be made at frequent intervals during the course of growth of the molds in question.

Only one type of corn was used as a substrate in these experiments. Small amounts of mold growth were observed on other varieties of corn, both soft and hard, but little poisoning resulted from feeding these varieties. In 1953, mold poisoning was diagnosed clinically in hogs eating hard, yellow, hybrid corn, indicating that the problem is not confined solely to soft corn. However, field observations indicated that the molds grew more often on the soft corn, such as used as the substrate in these experiments, and that swine would eat other corn, if it was available, in preference to moldy corn. Experiments with *P. rubrum* Stoll and the toxic strain of *A. flavus* Link on other varieties of corn have not been attempted.

The information available indicates that toxic substances produced by molds are relatively stable in storage,[2-5] and could increase due to continued growth of the molds. However, it is possible that metabolic interactions could reverse this process.

The data in tables 1 and 2 indicate that the Penicillium is the more toxic of the two molds. Approximately 7 lb. of the Aspergillus-corn substrate, fed over a four-day period, was necessary to produce the death of swine, with the exception of 1 pig. No attempt was made to produce acute poisoning by feeding larger doses. The minimum

lethal dose was not determined; however, 8 oz. caused the death of 1 pig in less than 18 hours.

The fact that 30 oz. of the Penicillium-corn substrate did not produce death, when fed over a 12.5-day period, indicates that the liver is able to detoxify small amounts of this toxin over a period of time. A "threshold effect" could also be operative, in that partial excretion of the toxic substance *per se* would not allow a sufficient "build up" with this particular dosage level and time interval. Apparently, the dose of the substrate required to produce acute poisoning in swine is less than 8 to 12 oz. The equivalent of 8 oz. of the experimental mold substrate could readily be consumed in a day by a pig while eating moldy corn in the field. Deaths occur three to 14 days after the animals have been turned into a field, beginning on about the sixth day.

The 24 oz. of Penicillium-corn substrate that proved toxic to the goat is probably in excess of the minimum lethal dose. The animal was very depressed when the second 12 oz. was administered.

The acute toxic dose of the Penicillium-corn substrate for horses appears to be about 4 kg. when given over a one-day period in the form of supernatant fluids. When approximately the same dosage was given in smaller increments, death occurred in five to 11 days instead of 34 hours. This indicates that the toxic material, whether it is in the spores or corn substrate, is water soluble.

Further work needs to be done with standardized corn substrate to establish a minimum lethal dose for these two toxic molds.

## SUMMARY

1) Thirteen cultures of mold were recovered from corn found in fields where hogs were dying. Two of the 13 cultures proved toxic when grown in a pure culture on autoclaved corn which was then dried, ground, and fed to swine.

2) The toxic molds have been identified as *Penicillium rubrum* Stoll and a strain of *Aspergillus flavus* Link.

3) Of the remaining 11 cultures, all of which were nontoxic, eight were strains of *A. flavus* Link and three were *Fusarium moniliforme*.

4) Experimental poisoning was produced in swine and mice with the toxic *A. flavus* Link and with *P. rubrum* Stoll. A goat and 4 horses were poisoned with *P. rubrum* Stoll.

5) The toxicity of the mold-corn substrates was unchanged by heating to 60 to 70 C. for 26 hours, or by storage in a cool, dry place for 61 days.

6) The toxic principle formed by the growth of *P. rubrum* Stoll on ground, dried corn was present in the aqueous supernatant extract, which was toxic for horses.

7) The gross histological lesions produced by the toxic molds are described.

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# Poisoning by Sodium Salt — A Cause of Eosinophilic Meningoencephalitis in Swine

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MANY ACCOUNTS of a clinical syndrome associated with the inclusion of excess salt in the diet of pigs, either accidentally or through ignorance, have appeared in the literature. In spite of this apparent causal relationship, numerous attempts to produce the characteristic clinical picture experimentally have been unsuccessful.[44]

Rasenack[21] fed sodium chloride mixed with fish meal to pigs, 2 of which developed signs of salt poisoning. Moore[28] produced a disease with neurological signs by feeding powdered soap containing up to 55.5 per cent sodium carbonate. Recently, Bohstedt and Grummer[4] have induced signs in pigs by increasing the sodium chloride (NaCl) and reducing the water intake.

In the past, salt poisoning in swine has been diagnosed clinically on the basis of a history of increased salt intake and the signs manifested by the animals. Often, no significant changes could be demonstrated at necropsy. Reference was made to gastroenteritis but the stomach and intestines were often normal. The stomach contents frequently failed to demonstrate an excess of sodium salts. Macroscopically, the brain was described as being edematous and congested. Prior to preliminary reports of the present study,[25, 26, 28] microscopic examination of the brain in this disease has not been reported. Hjarre,[13] Olafson,[27] and McIntosh and Smith[24] observed an encephalitis in pigs characterized by infiltration of eosinophils into the meninges and perivascular spaces. Hjarre and Olafson associated certain of these field cases with the feeding of brine or salted fish meal. Michalka[23] and, recently, Larson, Möller, and Thordal-Christensen,[16] and Yamagiwa[45] have described eosinophilic meningoencephalitis in pigs.

The present paper is an account of studies which were instituted in the fall of 1951 in an attempt to: (1) produce salt poisoning in pigs experimentally; (2) record the clinical manifestations of the disease; (3) study the gross and microscopic pathological changes of the disease in an effort to make exact diagnosis possible; and (4) determine the pathogenesis of the condition.

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## Materials and Methods—General

The source and type of pigs used are given in each experiment. The basic rations, except where otherwise indicated, were regular commercial pig feeds designated A and B.

Ration A consisted of wheat, screenings, corn, oats, barley, alfalfa meal, meat scraps, ground limestone (1.5%), and NaCl (0.5%).

Ration B consisted of dehydrated meals of cereal grass and alfalfa, bone meal, tankage, linseed oilcake meal or soybean oil meal, meat meal, limestone, defluorinated phosphate, manganese sulfate, iron sulfate, iodized salt (0.5%), irradiated plant sterol (vitamin $D_3$), fortified vitamin A and D oil, cobalt sulfate, distillers' solubles, barley or corn, oats, wheat bran, wheat shorts, wheat or screenings, vitamin $B_{12}$, and antibiotic supplement.

Blood samples were taken from the anterior vena cava according to the technique outlined by Hoerlein, Hubbard, and Getty.[14] Chloride in the blood stream was determined by the method of Schales and Schales.[29] Calcium levels were obtained according to the method of Clark and Collip.[7] In experiments 1 to 5, inclusive, levels of sodium in the serum were obtained by the colorimetric method of Stone and Goldzieher.[37] In the rest of the experiments, the levels of the sodium and potassium in the blood were established by the flame photometric method.[1] Urine chloride was determined semiquantitatively by the Fantus method,[9] and urine sodium according to the method of Goldzieher and Stone.[11] Randolph's[30] technique was used for the enumeration of eosinophils in the blood. Hemoglobin determinations and total and differential counts of leukocytes were carried out according to the standard techniques. Euthanasia was accomplished with pentobarbital sodium anesthesia and exsanguination. Perfusion of the brain through the carotid arteries was employed in a few instances; however, rapid fixation was obtained by removing the brain immediately, cutting the body of the corpus callosum, and placing the brain in pure formalin for 12 hours. It was then removed from the formalin and sections taken. The remainder of the brain was placed in 10 per cent buffered formol-saline solution. Frozen sections were cut from the fixed tissue. Except where otherwise indicated for the special stains, sections were prepared by the standard paraffin embedding technique, cut at approximately 6 $\mu$, mounted, decerated, carried back to water, and then stained with hematoxylin and eosin.

Special stains employed were scarlet red (Sudan IV) for fat,[12] Mallory's phosphotungstic acid hematoxylin as modified for formalin-fixed material for myelin and gliosis,[19] Mallory's analine blue col-

lagen stain (1936),[39] Cresyl violet for Nissl granules,[17] Penfield's modification of Hortega's method for microglia and oligodendroglia, Penfield,[39] and von Kossa's technique[17] for demonstrating inorganic material.

## EXPERIMENTAL PROCEDURES AND RESULTS

### EXPERIMENT 1

It was decided to feed salted fish and brine because: *(a)* many of the reports in the literature pertained to the feeding of pickling brine and salted fishmeal; *(b)* attempts to reproduce the classical neurological syndrome by the addition of salt alone to the diet had invariably failed.

*Materials and Methods.*—The 3, 2-month-old Yorkshire pigs, 2 males and 1 female, designated A, B, and C, which were used in this experiment, were fed part of a barrel of commercial mackerel in brine.

One of the male pigs (A) was penned and fed separately and was given increasing amounts of the salted fish and brine mixed with basic ration A. More NaCl was added later. The amounts of mackerel, brine, and salt were increased as rapidly as tolerated by the pig until day 29 when it was consuming 250 Gm. of fish, 300 ml. of brine, and 100 Gm. of pure NaCl (approx. 250 Gm./day). Water was given ad libitum for the first three days, then only in amounts necessary to get the pig to eat his daily quota of salty feed. Blood studies were made at intervals.

The control pigs, B and C, were fed the same basal ration as pig A but in larger amounts. All pigs were weighed periodically.

*Results.*—On the third day after the high salt diet was started (with water given ad libitum), pig A had a profuse diarrhea. When the water intake was restricted, the feces became dry and firm. Thereafter, the pig regulated its consumption of food according to the amount of water given so that, with the higher dosage of salted feed, toward the end of the experiment, it was necessary to give all the water it could drink several times per day. Shivering, treading, tenesmus, salivation, and passage of flatus followed consumption of large amounts of cold water. Polydipsia, polyuria, pruritus, and hyperesthesia were constantly exhibited by pig A. The skin became dry and parchment-like, with excessive exfoliation. Other than a slightly increased irritability commencing about the ninth day, there were no signs attributable to the central nervous system.

During the test, pig A had increased in weight from 12.2 kg. to 21.4 kg., pig B from 12.3 kg. to 20.0 kg., and pig C from 11.0 kg. to 19.0 kg.

The eosinophil, and total and differential leukocyte counts, hemoglobin determinations, and serum calcium levels were not significantly different in the test and control pigs. The serum chloride level was significantly increased to a high of 126.5 mEq. per liter on feeding day 22.

*Discussion.*—The gradual increase in the salted fish, brine, and NaCl content of the feed, even to high levels, did not result in salt poisoning.

There was the possibility that the brine and salted fish did not contain the toxins or poisons believed responsible for the nervous syndrome or that the clinical and pathological picture which we were trying to correlate with the feeding of salt was an allergic phenomenon since it was associated with the infiltration of eosinophils into the brain. It was postulated that, in the presence of excessive dietary salt, certain allergenic polypeptides from the fish or brine would be absorbed. A suggestion that degradation products of *Ascaris lumbricoides*, known to be extremely allergenic (Vaughn[40]), were involved in the development of the hypersensitivity was also considered.

### EXPERIMENT 2

The hypothesis of an allergic mechanism was examined, since many investigators had failed to produce the syndrome by simply feeding excess salt.

*Materials and Methods.*—The 3 pigs from the previous experiment were used. Pigs B and C were fasted for 20 hours and then given a large quantity of feed consisting of salted mackerel (500 Gm.), mackerel brine (600 ml.), and pure NaCl (50 Gm.)—a total of approximately 435 Gm. of NaCl in 800 Gm. of basic ration A.

The following day, 200 Gm. of fresh roundworms (*Ascaris lumbricoides*), from the intestine of a pig, were ground with 150 Gm. of NaCl in a mortar and pestle, mixed with the commercial hog ration, and given to the 3 pigs.

After 33 days, the 3 pigs were fasted for 24 hours and then fed salt mackerel (450 Gm.), mackerel brine (1,800 ml., approx. 787.5 Gm. NaCl), and basic ration A (1,500 Gm.) mixed with ascarids (500 Gm.) ground with 100 Gm. of salt and added to the mixture. They consumed all of it within 36 hours. The same feeding was repeated three times, but without the ascarids and the 100 Gm. of salt, and each was eaten in 48 hours.

The 3 pigs greedily drank 18 liters of water per day. Hemoglobin and total and differential leukocyte counts, and serum calcium, chloride, and

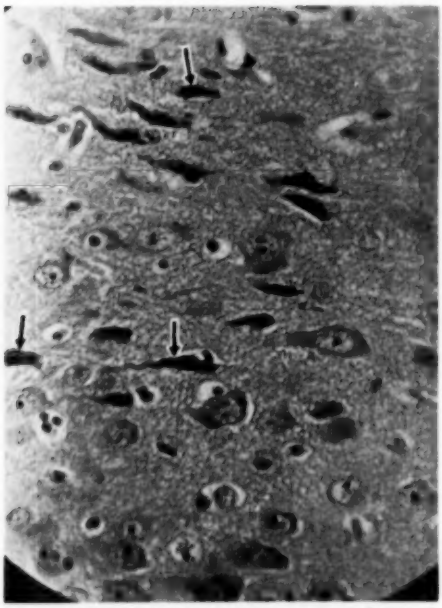


Fig. 1—Dark-staining angular neurones (arrows) in cerebral cortex of pig A; x 500.

sodium levels were established in pig A, on illness days 1, 2, and 4.

*Results.*—All 3 pigs appeared normal at noon on feeding day 7 and pigs B and C continued so, but at 1:15 p.m., pig A was on its left side, unable to rise, with limbs extended and quivering. A few minutes later, it commenced paddling with the movement of rapid running, twitching its facial muscles, blinking rapidly, and nystagmus was observed. When the running movements ceased, profuse salivation occurred and deep red, sharply demarcated maculae in the skin of the abdomen appeared suddenly, remained for 20 to 30 seconds, then disappeared. Flexor and crossed extensor reflexes were exaggerated. When placed on its feet, the pig was incoordinate and tended to stagger backward and fall. Following the attack, its temperature was 38.5 C., pulse and respiration rates, 100 per minute. The blood serum sodium level was increased and the eosinophil count in the blood dropped from 354 to 16 per cubic millimeter.

The pig remained on its right side with occasional paddling, twitching, and salivation interrupting extended periods of comparative quiet.

A second attack, similar to the first but more violent, occurred at 8:00 p.m. and lasted for several minutes. Involuntary twitching of the head and paddling persisted until 8:30 p.m. The pulse rate was 150; the temperature 40.3 C.

On day 2, a state of complete relaxation was punctuated at irregular intervals by seizures with twitching of the facial muscles, paddling, and nystagmus followed by rigidity and opisthotonos. Respiratory arrest and cyanosis were associated with the rigidity. The pulse and respiration were normal, except when increased during attacks, and the temperature was 39 to 40 C. throughout the day.

The animal did not respond consciously to external stimuli. However, spinal reflexes could be elicited readily and were considered to be slightly exaggerated.

At 4:00 p.m., when its nose was pushed into water, the pig made no attempt to drink and water poured into its mouth ran into the trachea, producing rattling râles but no coughing. The animal was turned from side to side during the day to prevent hypostatic congestion.

On day 3, there were two mild attacks, but toward evening, the pig manifested a degree of consciousness by a movement of

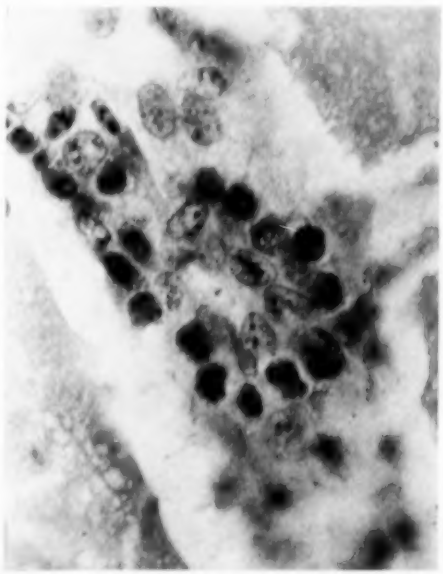


Fig. 2—Eosinophils surrounding blood vessel of pig A; x 500.

the head or limb nearest to the site of a slap. Food and water were ignored.

On the morning of day 4 the pig was on its feet, shakily walking, sniffing, and rooting in the straw. Its extreme nervousness, with sudden tenseness at any sound or contact, could be accounted for in part by the fact that it was blind. Although it appeared to be searching for food or water, both were avoided when its nose was placed in them. At 2 p.m. the pig was killed.

*Necropsy Findings.*—The carcass was dehydrated. The brain, spinal cord, and all other organs appeared normal.

*Microscopic Examination.*—The only significant changes were in the cerebrum and the overlying meninges. Many neurones in sections of the frontal area were dark, angular, and shrunken. The nucleus frequently blended with, and in some instances was indistinguishable from, the darkly staining cytoplasm (fig. 1). The remainder of the cortex showed similar nonspecific degenerative changes. The capillaries in, and the meninges covering, the cortex of the occipital and temporal lobes were infiltrated with eosinophils. Many of the capillaries had only a few of these cells about them while others, usually

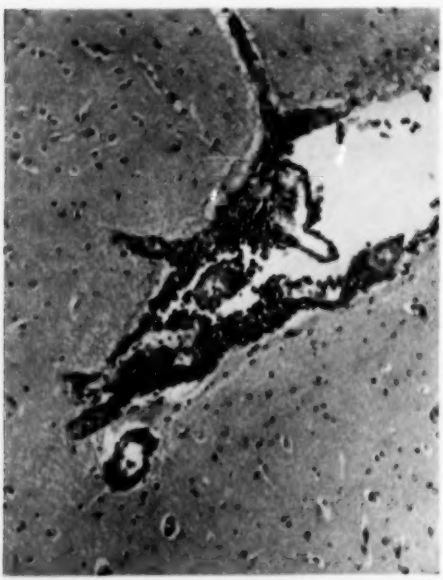


**Fig. 3—Meninges and perivascular space heavily infiltrated with eosinophils. The concentration of cells in the tip of the sulcus is characteristic. Pig A; x 120.**

**Fig. 4—Ulcers in the gastric mucosa of a salt poisoned pig. Pig B; x 500.**

closer to the surface, were entirely surrounded by one or two rows (fig. 2). A few round cells were also in evidence and the endothelial cells of the vessel walls were swollen and vesicular. In the meninges, the eosinophils tended to be concentrated in the depths of the sulci (fig. 3).

*Discussion.*—The sudden drop in eosinophil levels is a typical response to stress associated with the release of steroid hormones from the adrenal cortex.[32]

Although a characteristic clinical syndrome associated with eosinophilic meningoencephalitis had been produced, it was not possible to determine which constituent of the diet was responsible. The theory of a sensitivity was considered unlikely as the animal did not show any signs until seven days after re-exposure to the diet containing the ground ascarids, fish, brine, and large amounts of salt.

## EXPERIMENT 3

Since the diet in experiment 2 was complex, it was simplified, progressively, in the hope that the causal factor would become apparent. Basic ration A was considered innocuous as it was a widely used commercial feed. Since experiment 1 indicated that a pig fed gradually increasing amounts of salt could develop a tolerance to the higher levels, the basic ration plus a large amount of salt was fed initially.

*Materials and Methods.*—Pigs B and C, used in experiment 2, were fed basic ration A for three weeks. Chemically pure NaCl was then mixed with the 1,200 Gm. of the dampened ration and fed daily. The salt was mixed and given at the rate of 350 Gm. the first day and 400 Gm. on the next

two days. Each day, 12 liters of water was supplied at intervals timed to stimulate maximum food consumption.

Blood samples were not taken until pig B showed signs of illness, since the excitement of handling might have decreased food consumption. Samples were then taken from both pigs daily. The trough space was reduced so that competition induced them to eat more of the unpalatable feed.

*Results.*—The pigs ate the salted feed readily at first but 400 Gm. of the morning feeding was still in the trough when illness first appeared in pig B on the afternoon of the third day. Pig C remained well.

On day 1,* pig B appeared dazed and incoordinated but drank, ate, and was responsive to external stimuli at 5:30 p.m. By 11:30 p.m., it was lying on its sternum with forelegs extended and head retracted. When placed on its feet, it walked in tight circles to the left, hitting its head and nose on objects in its path, or catching its lower jaw on the edge of the trough. It lacked the intelligence to back or turn its head to free itself from the obstructions.

At 2:30 a.m. of day 2, the pig collapsed, apparently comatose. When lifted to its feet at 3:30 a.m., it stood stiffly with the head raised and twitching upward rhythmically, 60 times per minute. With each twitch, the eyes blinked and both ears jerked downward slightly. The twitching suddenly increased in frequency and severity and the pig moved sideways, looking back as though some feared object was approaching its side. It then stopped and began jerking its head directly upward, assuming a position similar to that of a sitting dog, and pushing backward with its forelimbs. The jerking of the head stopped suddenly, the facial muscles became tense, cyanosis developed, the mouth opened and released a copious flow of saliva. The pig fell on its side, was motionless for a few minutes, then opened its eyes, stood up, and resumed its circling to the left. Its pulse was 100 and pounding, respiration 12 per minute, reflexes were accentuated and pupils dilated.

The attacks became more frequent and severe, the intervals between them varying from seven to 21 minutes. They commenced as described above but the pig would move backward rapidly and fall on its side or sit like a dog, pushing with the forelegs and occasionally turning a backward somersault.

From 3:30 p.m. on, violent seizures recurred at regular seven-minute intervals.

After 2:00 a.m. of day 3, the pig was observed only during periods of one half to one hour. The fits recurred every seven minutes with some variation in severity but not in pattern.

On day 4, seizures continued at seven-minute intervals, characterized by head movements, twitching of the nose, respiratory arrest, and cyanosis; usually followed by coma until the next attack. Occasionally the pig would get up and walk in a circle, pushing against any obstruction. When water was poured into its mouth it would swallow convulsively as the water reached the pharynx. About a pint was consumed in this way.

On day 5, convulsions continued at intervals of seven minutes, each heralded by twitching of the nose followed by twitching of the facial muscles and clonic spasms of the neck which jerked the head backward repeatedly.

On day 6, no fits occurred from 3:15 to 4:00 a.m. The pig did not respond to external stimuli and collapsed immediately when placed on its feet. At 9:00 a.m. it was up and walked aimlessly for four hours, apparently blind. It attempted to drink water but the act was not well coordinated.

The pig was killed at 10:00 a.m.

When pig B became ill, its eosinophil count had decreased to 180 per cubic millimeter; within 24 hours it was zero. No other significant change occurred in the hemogram.

The levels of calcium in the serum in both pigs had increased during the period of high salt feeding from about 5.0 to 6.9 mEq. per liter. In pig B, the levels of sodium were increased by about 12 mEq. per liter shortly after the onset of illness, and it increased by 20 to 170 mEq. per liter by day 6.

*Necropsy Findings (Pig B).*—The animal was dehydrated. The intestines were empty. The gastric mucosa presented three irregular ulcers on the greater curvature (fig. 4). The other organs, including the brain, showed no gross changes.

*Microscopic Examination.*—The subarachnoid space over the cerebrum (fig. 5) and the pia accompanying the blood ves-

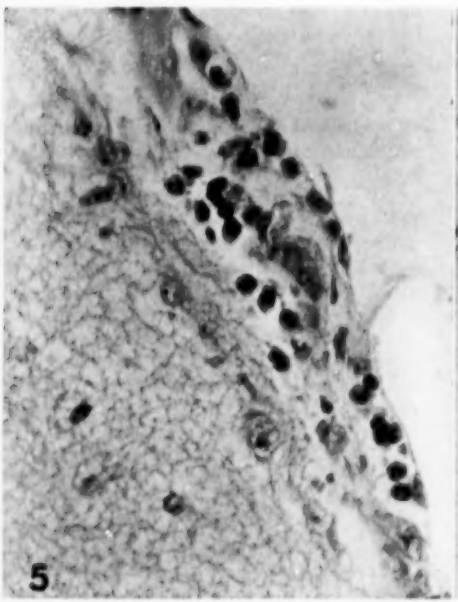
*Days are numbered from, and include, the day of onset of illness.

sels into brain substance (fig. 6) contained many eosinophils, particularly at the tips of the sulci.

Numerous vacuoles, in the zone corresponding to Brodmann's lamina 6, varied in size up to 40 $\mu$ (fig. 7). Laminae 3 and 4 presented capillary proliferation, malacia (fig. 8), and an increased cellularity (fig. 9), some of the latter due to the eosinophils. Most of the cells, however, appeared to be derived by proliferation (mitotic figures were in evidence) of cells from blood vessel walls. Many microglia could be identified (Penfield's stain [29]) by their processes which were becoming shorter, thicker, and vacuolated. In these areas, neurones were absent or appeared as deep pink-staining globules with none or only a part of a nucleus remaining. Neuronophagia was actively in progress. Gliosis could not be demonstrated; however, a minimum of six days is considered necessary for gliosis to become apparent. The severely damaged tissue was most evident in the third, fourth, and sixth laminae (less in the fifth) of the walls of the cortical sulci.

Changes were least advanced in the prefrontal cortex, became progressively more severe to the parietal and temporal lobes, then diminished to the occipital cortex. In the most severely affected areas, foci in the cortical tissue adjacent to the lateral and central sulci showed almost complete disintegration with vacuolization, capillary proliferation, malacia, and some deposition of calcium (fig. 10). Nerve fibers and endings, as shown by Bodian's method,[17] were swollen and broken, and many had disappeared from the damaged area (fig. 11). Irregularly swollen "clubbed" axon cylinders were present in the corona radiata (fig. 12). Significant changes were not observed in the basal ganglia, thalamus, cerebellum, or brain stem. By means of Mallory's phosphotungstic acid hematoxylin stain, beading of the myelin sheaths and an apparent reduction in their numbers was demonstrated in the vicinity of the lesions in the cortex. The myelin tended to break into the central space. This stain proved to be selective for eosinophils (fig. 6) and was superior to some stains which are generally recommended for this purpose.

*Discussion.*—The most interesting feature of the hemogram was the rapid drop in eosinophils, from the normal number of 545 to 0/1 cmm. a few hours after the on-



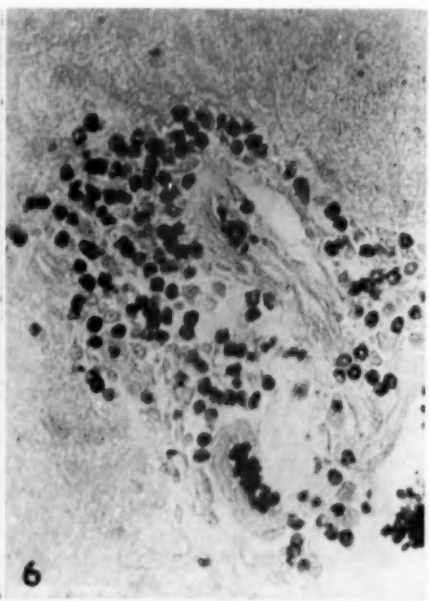




Fig. 5—Eosinophils in the subarachnoid space overlying the cerebral cortex of pig B; x 500.
Fig. 6—Eosinophils in pia and around blood vessels at inner tip of sulcus. Stained with Mallory's phosphotungstic acid hematoxylin stain; x 350.

set of illness. This phenomenon was also noticed in pig A (exper. 2).

We have no explanation for the extensive symmetrical damage to the cerebral cortex.

### EXPERIMENT 4

In a preliminary trial, the pig refused to eat a mixture of salt with oat and barley chop but with no animal protein. Therefore, the procedure followed in experiment 3 was repeated with pig C alone.

*Materials and Methods.*—Pig C was fed basic ration A for several weeks in an attempt to avoid a tolerance for increased salt intake. Pure NaCl (10 to 13% of ration) was then fed. The pig ate 2,680 Gm. of NaCl in 13 days (av. 230 Gm. per day). Water was given at intervals in accordance with the intake of salty feed.

Blood and urine samples were collected at intervals for analysis. The animal was killed for necropsy after 18 days.

*Results.*—After 8 days, the serum sodium level was 160 mEq. per liter and the chloride and calcium levels increased. The urine contained 181.6 mEq. of sodium and 129.9 mEq. of chloride per liter. The blood cell counts and hemoglobin levels remained within normal limits. The daily water intake of 13 liters was approximately 11 liters above normal.

The pig exhibited polyuria and diarrhea during the period of high salt intake but no neurological signs of salt poisoning. Its weight increased from 160 to 180 lb. during the experiment.

At necropsy, there were no gross lesions and no microscopic changes in the brain or other tissues.

*Discussion.*—This pig consumed approximately the same amount of salt per day, for an extended period, as had pigs A and B when they developed neurological signs and lesions. The serum sodium level was approximately the same after eight days as that of pig B on day 1 of illness, indicating that factors other than the intake of NaCl and the serum sodium level are of significance in the production of the disease.

Experiment 4 differed from experiment 3 in that pig C was alone and had plenty of trough space. With no incentive for competitive eating, it was necessary to give large amounts of water before it would eat. The water was obviously utilized as a vehicle to eliminate large quantities of the NaCl. It is unlikely, therefore, that a high sodium or chloride concentration was maintained in the blood stream.

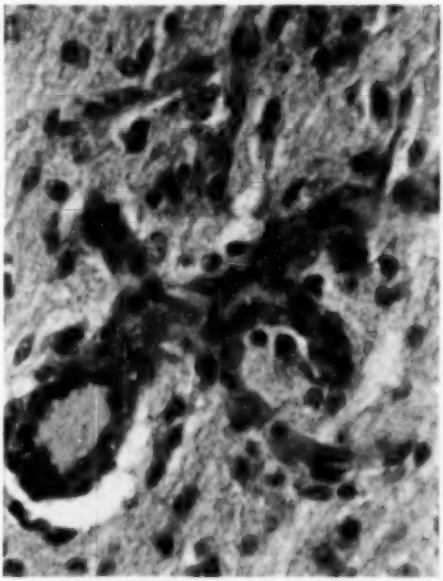


**Fig. 7—Selected area from fig. 8 showing capillary proliferation and increased cellularity; x 500.**

### EXPERIMENT 5

This experiment was designed to study the effect of high NaCl feeding on younger pigs.

*Materials and Methods.*—Four female crossbred Yorkshire pigs, 7 weeks old, were given water ad libitum and dry basic ration A, 3 to 4 kg. per day for nine days. The pigs were in poor condition, were not eating or growing well, and 2 of them had diarrhea.

On each of the next nine days, they were given milk (6 liters), water (4 liters), basic ration A (3 kg.), and vitamin $B_{12}$-chlortetracycline supplement (2 Gm.). The milk and vitamin $B_{12}$ supplement were omitted for the next seven days and 5 kg. of the basic ration was fed mixed with 2 liters of water. An additional 6 to 8 liters of water was consumed each day.

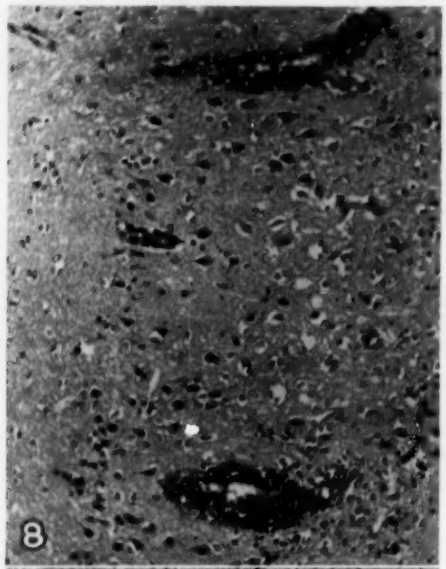
The pigs made steady improvement on the supplemented diet. They were weighed when placed on test and again before death.

Blood samples were taken, always between 4:00 and 5:00 p.m., at four days and one day prior to the test, and at intervals during the high salt feeding. Water was withheld for four hours before the samples were taken in the hope that maximum levels of sodium and chloride could be detected. NaCl was added to basic ration A at the

rate of 10 per cent. The amount of water given depended on the rate the feed was eaten (table 1). After noon of the fourth day, no additional salt was fed. Pigs 1 and 3 were treated with dextrose, calcium, and magnesium solution on days 5 and 6, respectively.

Necropsies were conducted on all pigs after death or slaughter.

*Results.*—At 11:00 p.m. on the second day, after 39 hours on the high salt diet, pig 4 showed signs of illness, pushing its head against the trough. It suddenly began to jerk its head upward at regular intervals (36 times per minute) with increasing violence until the whole forepart of the body, as well as the ears, were jerked upward at each movement. Later, the head was flexed tightly to the left, along the shoulder (pleurothotonos) and the pig pivoted to the right on its right forefoot, keeping the left forelimb straight. Toward the end of this movement, the nose was held to the ground as though being used as an additional support. The pig was not conscious. Similar attacks occurred at irregular intervals until midnight when the pig became prostrate. When observed at 3:00 a.m., a seizure occurred, terminated by rigidity, respiratory arrest, and cyanosis; the temperature was 39.5 c., the pulse was 120 and thready. The pig died at 7:00 a.m., 47 hours after salt feeding began.



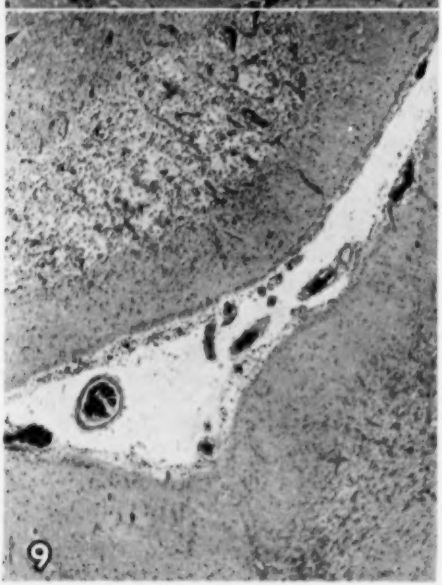




**Fig. 8—Perivascular infiltration with vacuolation and increased cellularity in cerebral cortex; x 120.**

**Fig. 9—Malacia of cerebral cortex with capillary proliferation. Note position of lesion in relation to sulcus; x 40.**

*Necropsy Findings.*—The skin was deep purple, darkest on the ventral surface of the body. The mucosa of the pyloric region of the stomach was reddened and edematous. A few roundworms were present in the jejunum and the large bowel was filled with dry, coarse residue.

*Microscopic Examination.*—All of the cellular elements of the cerebral cortex, including the neurones, were separated by a space from the surrounding tissue (fig. 13) The pyramidal cells were frequently forced into the form of a crescent at the side of a vacuole "signet ring" or were suspended by fibrils, axones, and dendrites bridging the distended perineuronal space (fig. 14).

Numerous vacuoles, varying in size singly or in rows, were in all layers of the cerebrum. They were usually rounded but, in the white matter, tended to be elongated. Patchy edematous vacuolation formed cystic structures which gave a lacy appearance to the tissue (fig. 15).

The Virchow-Robin spaces, like the perineuronal spaces, were greatly dilated. Eosinophils had infiltrated, taking up a position in the meninges at the inner tip of the sulcus and just outside the endo-

thelium of the capillaries of the molecular, granular, and pyramidal layers of the cerebral cortex (fig. 13). The hippocampus and subcerebral portions of the central nervous system, including the cerebellum, showed no lesions.

At noon of the third day, 59 hours after the test began, pig 1 appeared to be blind. Periodically, the nose twitched rapidly and the head was jerked upward in clonus of the neck muscles. It tended to stagger sideways to the right with its head pulled to the left side. Pig 3 appeared to have impaired vision and paid little attention to its surroundings. Seven liters of the water allowed for the third day was given at 5:00 p.m. Pig 2 drank readily, pig 3 drank somewhat more slowly, and pig 1 was not interested until its snout was pushed down into the water, when it drank clumsily.

At 6:00 p.m., pig 1 started to have attacks ushered in by twitching of the nose and clonus of the neck muscles resulting in a "woodpecker" movement of the head. The whole body was jerked backward, at times, in coordination with the head movement. The feet remained stationary and the center of gravity was shifted to the rear, thus the pig occasionally fell to a sitting position. When an attack terminated, it would resume an aimless, restless wandering. When it bumped into an object, it turned aside and continued walking.

The attacks were periodic but occasionally so mild as to fuse with incoordinated movements and random twitching of muscles which occurred between the more defined seizures.

At 9:00 a.m. on the fourth day, pigs 1 and 3 were both having attacks at regular seven-minute intervals. A frightened expression and a period of expectant, alert immobility in the standing position with the nose just above the ground portended a seizure at this stage. This attitude was terminated when the nose began to twitch. The head was pulled around to the side tightly, then returned to the forward position to start jerking backward with the rest of the body. The pigs pivoted around one front foot, and dragged the other in the manner previously described. Frequently, they propelled themselves backward in a sitting position by working the back legs rapidly while the forelimbs were held rigid much like a horse backing a heavy load.[28] When, in this position, the head jerked backward, the neck muscles were in clonus, and the mouth opened and

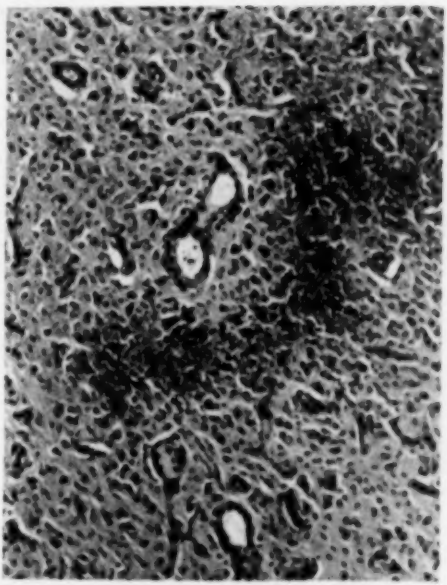


Fig. 10—Severely damaged area in the cerebral cortex of pig B; x 120.

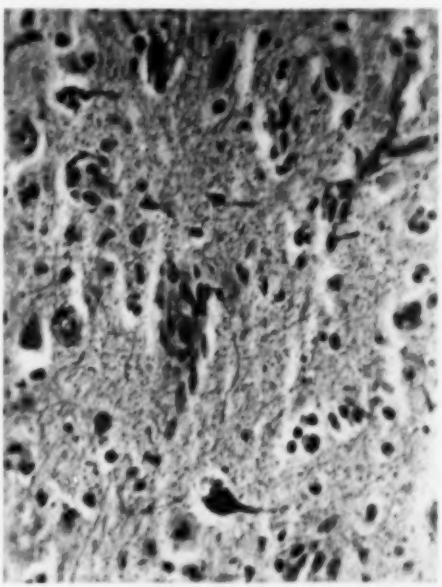


Fig. 11—Disintegration of nerve fibers. Stained with Bodian's stain; x 350.

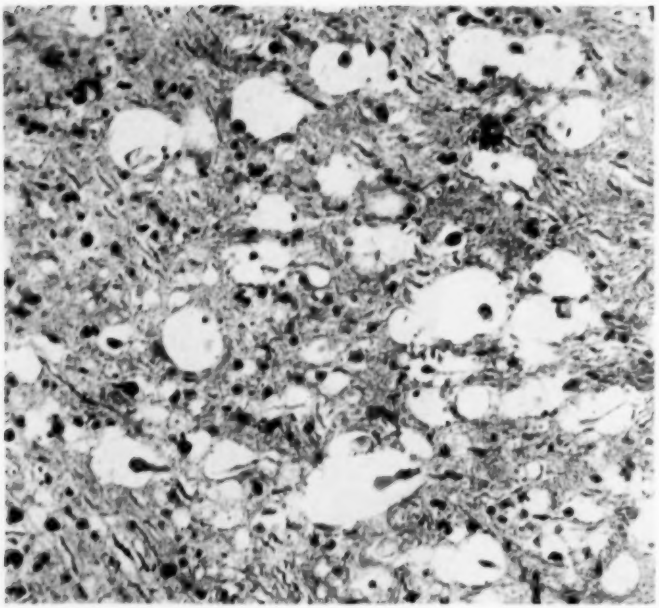


Fig. 12—Irregular swelling of the axone cylinders. Stained with Bodian's stain; x 500.

closed. Profuse salivation and dilation of the pupils terminated these attacks. The hyperactivity terminated in rigidity with respiratory arrest and cyanosis. Suddenly, the rigidity changed to complete flaccid relaxation for variable periods.

An attack could not be induced by external stimuli. During the intervals between attacks, the pigs were completely oblivious to surroundings. When placed on their feet, they would collapse as though boneless.

During the morning of the fourth day, pig 2 appeared to be blind and, although it responded vaguely to external stimuli, it showed no interest in or fear of anything. Only two attacks were observed in this pig. These were heralded by twitching of the nose, followed shortly by violent clonic spasms of the muscles of the whole body. The rump was jerked forward as in copulation. The muscular spasms in the second seizure terminated in the animal jumping backward in a series of stiff-legged hops.

Pig 2 showed some improvement in the afternoon and ate and drank some of the basic ration and water with no salt added. Pigs 1 and 3 were not aware of the presence of either food or drink but swallowed water that was poured into their mouths.

In the evening of the fourth day, the seizures in pigs 1 and 3 were less severe and they walked continuously and hurriedly around the pen between attacks. Occasionally, one or the other would stop and, with nose to the floor, would pivot around one foreleg.

Between 1:00 a.m. and 3:00 a.m. of the fifth day, pigs 1 and 3 each had 18 seizures. At 3:00 a.m., pig 3, immediately after a seizure, was given 30 cc. of a solution of dextrose, calcium, and magnesium intraperitoneally. It remained apparently blind and unaware of its surroundings but it had no more fits. Pig 1 continued having attacks regularly at seven-minute intervals and wandered aimlessly around, between attacks, for the rest of the day.

At 9:00 a.m., pig 3 walked around continually with its nose to the floor so that it pushed straw. The straw which collected in its mouth was chewed, apparently more by accident than by conscious effort. It ignored water and feed.

At 12:30 a.m. of the sixth day, pig 1 was still showing the typical clinical picture. Pig 2 appeared normal, ate, and drank but seemed unable to anticipate danger. Pig 3 drank and ate clumsily, was still somewhat incoordinated, but scratched

itself normally and was conscious of surroundings.

Attacks suffered by pig 1 were still characterized by nose twitching, head jerking, and backward movement, in a sitting position, propelled by the hindlegs with forelegs held stiffly. The nose pointed downward at the beginning, was raised stepwise and pointed upward with the mouth open and the ears back as the attack progressed. Occasionally, all four limbs were used to propel the animal backward rapidly. Salivation, pupillary dilation, rigidity, and cyanosis occurred late and, finally, the pig collapsed completely. Usually it remained in comatose collapse until the twitching of the nose signalled the onset of another seizure. Rarely, it walked aimlessly between fits, forcing its head and nose against any object it encountered.

Nine seizures occurred in pig 1 between 12:25 a.m. and 1:25 a.m. Immediately after the ninth seizure, 30 cc. of dextrose, calcium, and magnesium solution was administered intraperitoneally. Although another 20 cc. of the solution was given an hour later, seizures continued at seven-minute intervals until 6:00 p.m., when they ceased abruptly. The final attack was typical and equal in severity to the others. For the remainder of the day, the pig was weak and wandered about rooting in the straw, pushing its head against objects it encountered. It showed no interest in food.

On the seventh day, pig 1 rapidly regained its faculties. It drank greedily but awkwardly. Some of the water was aspirated and caused coughing.

On the eighth day, all of the pigs were essentially normal except that pigs 1 and 3 appeared to be blind. Euthanasia was performed.

Just prior to the test, pigs 1, 2, 3, and 4 weighed 22.7, 16.6, 18.0, and 16.1 kg., respectively. Before being killed, pigs 1, 2, and 3 each weighed 18.2 kg.

*Necropsy Findings.*—In pigs 1, 2, and 3, no marked changes were observed at necropsy.

*Microscopic Examination.*—The significant lesions in the central nervous system of pig 1 were confined to the cortex of the cerebral hemispheres and the overlying meninges.

Eosinophils had infiltrated the leptomeninges, tending to concentrate in these membranes as they dipped into the sulci

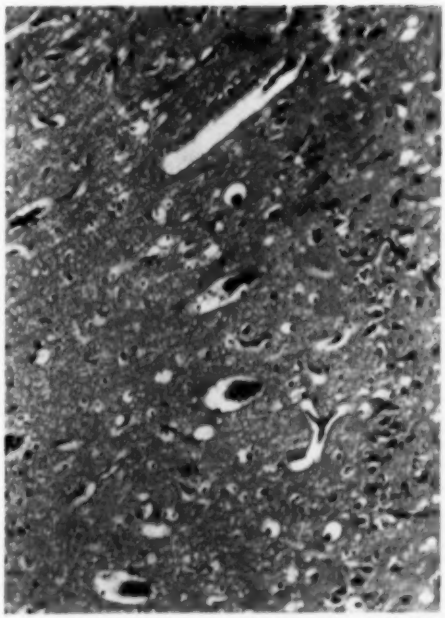


Fig. 13—Perineurone vacuolation with distention of perivascular spaces in pig 4; x 120.

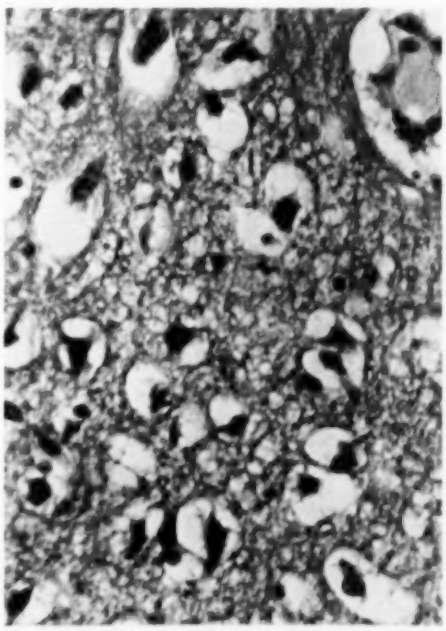


Fig. 14—Fibrils (axones and dendrites) bridging the distended perineuronal spaces in pig 4; x 500.

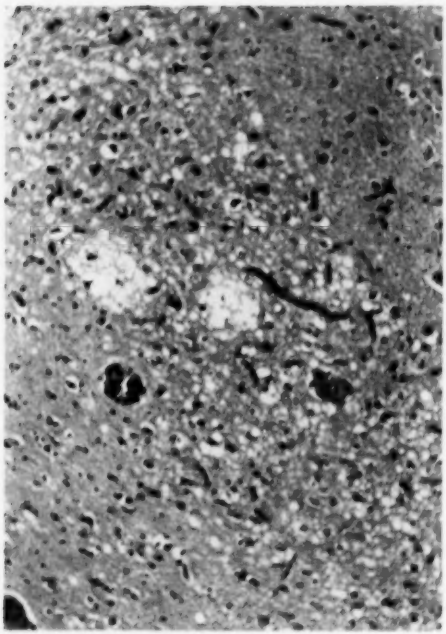


Fig. 15—Patchy edematous vacuolation in multiform layer of the cerebral cortex of pig 4; x 120.

Fig. 16—Numerous eosinophils surrounding blood vessels and in depth of sulcus of pig 2; x 120.

(fig. 16); few were present on the surface of the gyri. Eosinophils were also present in the perivascular spaces (fig. 16). The nuclei of endothelial cells appeared swollen and vesicular amid the eosinophils. Neocapillaries were surrounded by eosinophils in some areas, giving a highly cellular appearance to the tissue.

The most important changes were degenerative lesions exhibiting a topographic dominance primarily involving that part of the cortex which forms the walls of the sulci. The severity of the encephalopathy varied from simple, nonspecific neuronal degeneration characterized by shrinkage and pyknosis, seen in the precentral area, to complete destruction of the cortical tissue apparent in the walls of some of the sulci in the parietal, temporal, and occipital lobes. In the latter areas, the tissue presented many spaces which were bridged by capillaries and populated with glial cells, fat granule cells, and a few eosinophils (fig. 17). The ganglionic and fusiform layers of the cortex were invariably involved in the affected areas. Usually, however, changes could be found in the granular and pyramidal layers even in the less severe lesions. The molecular layers rarely showed any obvious changes.

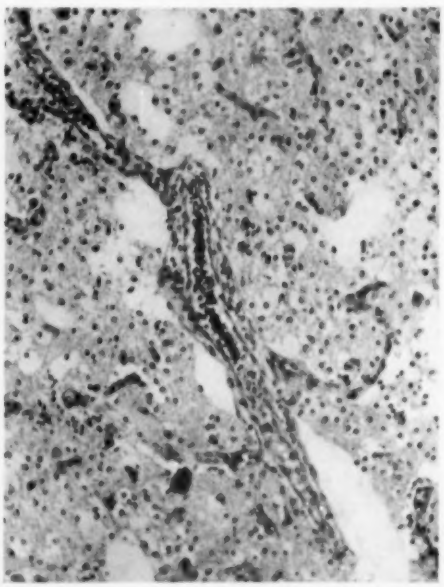


Fig. 17—Area of malacia in the cerebral cortex of pig 1. Almost complete disintegration has occurred. Note fat granule cells and capillary bridging spaces; x 120.

Proliferation of endothelial cells with numerous neocapillaries was a feature of the changes seen in all affected areas.

Only that part of the cerebral cortex that is formed from the archipalium, the uncus, and adjacent part of the hippocampal gyrus was unaffected. The cerebellum, basal ganglia, cranial nerve nuclei, and the various mesencephalic nuclei, including the red nucleus (fig. 18), were normal.

The changes in the brains of pigs 2 and 3 were essentially the same as those in pig 1.

Except for reduction of the number of eosinophils at the time of onset of illness, the hemogram was not remarkable. The results of analyses of the blood serums are presented (table 2).

*Discussion.*—The apparent ease with which signs of toxicosis were produced would suggest that pigs of 3 months of age are quite susceptible.

In this experiment, the pigs were prepared for the test by excessive feeding: (1) to minimize the possibility of the lack of essential nutritional factors, especially B-group vitamins and calcium, entering into the picture, and (2) to favor the development of a large appetite. The pigs were in excellent condition and had ravenous appetites when the test commenced. The competitive influence provided by limitation of trough space further stimulated food consumption. Water was given during the test in larger quantities but at longer intervals than in previous tests. This may have had some influence on the success of the experiment.

The seizures observed were basically the same as those seen in the previous tests. Thus they appear to have definite diagnostic significance. The frightened attitude and period of expectant immobility which signaled an attack in pigs 1 and 3 could be compared to the aura of idiopathic epilepsy in man and the dog.

The severe edema, demonstrated microscopically in the brain of pig 4 which died only eight hours after the onset of illness, suggests that the first change, and perhaps the immediate cause of these signs, is cerebral edema.

The eosinophil infiltration present in the brains of all pigs and the similarity of the lesions in pigs 1 and 2 to those seen in pigs A and B suggest that these lesions are pathognomonic.

Cessation of seizures in pig 3 after being given dextrose, calcium, and magnesium solution seemed to indicate a therapeutic effect. However, seizures have ceased abruptly in many other animals suffering from this disease. When pig 1 was subjected to the same treatment, no beneficial results were evident.

The lack of fear, blindness, and inability to perform simple actions, such as eating, when the pigs began to recover consciousness can be explained on the basis of the damage to the cerebral cortex. Their rapid recovery was remarkable when the amount of cortical damage is considered.

**TABLE 1—Daily Diet of Pigs 1, 2, 3, and 4 (Experiment 5) and Its Toxic Effect**

| Time from start of exper. (days) | Amt. basal ration (kg.) | Amt. water (liters) | Amt. milk (liters) | Amt. NaCl (Gm.) | Remarks |
|---|---|---|---|---|---|
| 1 | 2.6 | 4 | 3 | 260 | All pigs normal. |
| 2 | 1.3 | 6 | 3 | 130 | Pig 4 showing signs of illness. |
| 3 | 1.5 | 11 | 3 | 150 | Pig 4 died; Pigs 1 and 3 showing signs of illness. |
| 4 | .... | .... | .... | .... | Pig 2 showing signs of illness. |
| 4-8 | | | | | Animals given regular basal ration A and water with no extra salt until killed. |

**TABLE 2—Analytical Data on the Serums of 4 Pigs Poisoned with NaCl (Experiment 5)**

| Time from start of exper. (days) | Pig (No.) | Blood serum | | |
|---|---|---|---|---|
| | | Ca (mEq./liter) | Cl (mEq./liter) | Na (mEq./liter) |
| −4 | 1 | 5.8 | .... | 145.6 |
| | 2 | 5.7 | .... | 135.0 |
| | 3 | 5.9 | .... | 144.6 |
| | 4 | 5.8 | .... | 141.3 |
| −1 | 1 | 6.0 | 108.0 | 153.4 |
| | 2 | 5.9 | 102.5 | 157.3 |
| | 3 | 6.1 | 99.0 | 149.5 |
| | 4 | 6.3 | 104.0 | 153.4 |
| 2 | 1 | 6.4 | 133.0 | 186.1 |
| | 2 | 5.7 | 143.0 | 180.5 |
| | 3 | 5.7 | 128.5 | 174.7 |
| | 4 | 6.0 | 126.5 | 188.1 |
| 3 | 1 | 5.9 | 127.0 | 188.1 |
| | 2 | 5.6 | 133.0 | 188.1 |
| | 3 | 6.0 | 130.5 | 174.7 |
| | 4* | .... | .... | .... |
| 4 | 1 | 5.7 | 108.0 | 157.7 |
| | 2 | 5.8 | 109.0 | 135.9 |
| | 3 | 5.3 | 104.5 | 140.8 |
| 5 | 1 | 5.4 | 112.5 | 161.5 |
| | 2 | 6.0 | 98.5 | 154.0 |
| | 3 | 5.3 | 105.0 | 165.2 |
| 6 | 1 | 5.9 | 119.5 | 161.5 |

* Pig 4 died on day 3.

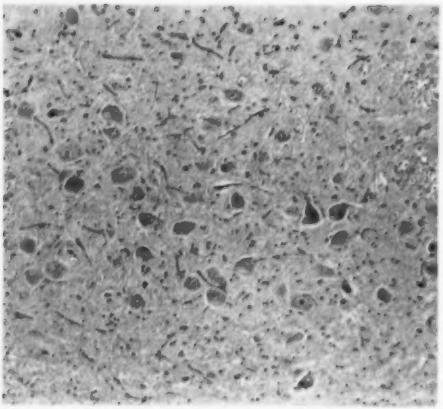


Fig. 18—Red nucleus in pig 1. Nerve cells and supporting tissue appear normal; x 85.

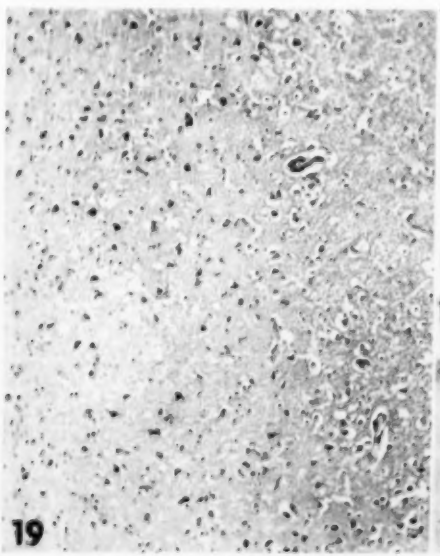
The number of eosinophils in the blood was reduced significantly during the period of stress. Blood serum calcium levels were not significantly altered during the period of salt feeding.

Serum chloride and sodium levels were markedly increased and roughly parallel. This suggests that these values may be of significance in making a differential diagnosis. By taking the blood samples at the same time each day, comparable results were obtained. The maximum serum sodium level recorded appears three times during the period of high salt feeding. This could be an indication that, when the blood serum sodium level is 188.1 mEq. per liter, the threshold of the intestinal absorption has been reached.

## EXPERIMENT 6

A survey of the feeding methods of swine raisers revealed that many mixed the solid food, garbage, grain, or commercial pig feed with water and fed it in the form of swill. In many instances, only one trough was available and the only fluid that the pigs received was that which was mixed with the feed. This appeared to be a dangerous practice in the light of our findings, since the feed, particularly garbage, could readily contain considerable NaCl. A separate unlimited supply of fresh water seemed to be the logical method of guarding against such a contingency. Experiment 6 was planned to test this prophylactic measure.

*Materials and Methods.*—Ten Yorkshire pigs, 3 months old, were fed basic ration B for several weeks preceding the test. A blood sample was taken from each pig and, after 24 hours of fasting,



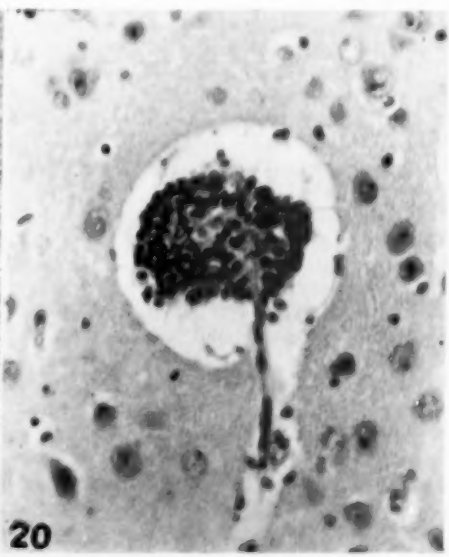




Fig. 19—Distended pericellular and perivascular spaces in pig 9; x 85.
Fig. 20—Eosinophils in distended perivascular space in pig 43, which was given sodium propionate; x 350.

they were weighed, and two groups of 5 pigs each were placed in pens numbered 3 and 4.

The pigs in both pens were fed swill composed of NaCl (2.0%), basic ration B (24.5%), and water (73.0%).

Pigs 10 to 14 in pen 3 had free access to fresh water until the sixth day, after which additional water was withheld. The salt swill was removed at 10:00 a.m. on day 9 and the pigs were given their regular ration of swill (basic ration B—30%, bran—4%, and water ad libitum).

Pigs 5 to 9 in pen 4 were given additional fluid by further diluting their swill only when they would not eat it otherwise. This was done on days 2 (2.5 liters of water), 4 (3 liters of water), and 5 (4 liters of milk). On day 6, the 3 remaining pigs (5, 7, and 8) were returned to the regular ration (table 3). Blood samples were taken at intervals for chemical analyses (table 4); those for chloride were taken separately and analyzed immediately; those for sodium and potassium were placed in airtight screw-cap vials and frozen until they could be analyzed by flame photometry.

On day 6, the 3 remaining pigs in pen 4 were weighed and blood samples were taken.

*Results.*—On day 1, after 24 hours' fasting, the weights of the 10 pigs ranged from 19 to 42 kg.

On day 3, pig 6 was in considerable respiratory distress from a hematoma, the result of bleeding, so it was killed at 9:00 a.m. Pig 9 was wandering around aimlessly. It responded to noises and contact but appeared to be blind and stupid. All other pigs were normal.

At 8:00 a.m. of day 4, pig 9 was weak and had several well-defined, typical seizures during the next two hours. Between attacks, it staggered around bumping into and pressing its head against objects. It was unconscious and completely oblivious to external stimuli. At 10:00 a.m., it went down on its side and remained there. Its head jerked upward and its forefeet moved in a running motion continuously until it died 16 hours after the first signs were observed and 75 hours after first receiving the high salt feed.

Pig 8 was blind and responded sluggishly to physical contact.

At 9:00 a.m. on day 5, pigs 5, 7, and 8 appeared blind. The neck muscles of pigs 7 and 8 showed mild clonus, the heads jerking slightly backward (approx. 90 times per minute.) These pigs drank from the surface after 4 liters of water was mixed with their feed at 9:00 a.m. They ate little of the salt feed.

The pigs in pen 3 ate all their feed during the day.

On day 6, pigs 5, 7, and 8 were severely dehydrated. Their skin was dry and parchment-like. They had lost 9.3, 4.0, and 3.3 kg. in weight, respectively, in the six-day period. Pig 8 appeared to be more severely affected clinically, so it was killed.

Pigs 5 and 7 were given regular feed and water which they took reluctantly at first, but they were apparently normal in every respect four days later.

The normal sodium and chloride serum levels and the appearance of pigs 10 to 14, which had been given additional water, indicated that they were in good health. The high salt diet was continued but the additional water was taken away at 10:00 a.m. of day 6 and withheld until day 9.

On day 9, the pigs were less lively but otherwise normal. It was decided to discontinue the experiment. The salted food was replaced at 10:00 a.m. and the pigs consumed the fresh water and feed readily.

At 8:00 a.m. of day 10, 22 hours after the salt feed was replaced, pigs 12 and 13 were unconscious and undergoing epileptiform attacks typical of those previously described. Pig 12 occasionally ran forward or backward and bumped into objects and the fits were frequently terminated by copulatory thrusts. Between attacks it walked, stepping high with the front feet, or rooted with urgency in the soft ground. The attacks came at irregular intervals, one series being timed with 7-, 14-, 7-, and 18-minute intervals. Pig 13 also tended to step high and to root. Its nose twitched continuously but this became more apparent just before clonus of the neck muscles and forced circling which occurred at irregular intervals.

At 2:00 p.m., pig 12 was down on its side panting; all four legs were rigidly extended. Its body temperature was 41.7 c. It was thought that the animal was suffering from "heat stroke" (the atmospheric temperature and humidity were high), and from hyperventilation. By pouring cold water over it, the temperature was reduced to 39.3 c., the respirations slowed, and the rigidity gradually lessened. The pig remained comatose, and died at 4:00 p.m.

Pig 13 continued its activities until, at 5:00 p.m., it was standing rigidly, head lowered, panting, and with a temperature of 41.1 c. Since it would not drink, water was poured over it. The temperature was reduced, normal breathing was resumed,

and involuntary movements ceased. However, at 7:29 p.m. a typical attack terminated when the pig fell over on its side and became rigid with respiratory arrest and cyanosis.

At 7:45 p.m. a second fit occurred. The whole body was jerked backward violently, eliciting a grunt at each jerk. This attack lasted two minutes and a third fit occurred 11 minutes later.

On day 11, seizures continued to occur in pig 13 at irregular intervals with diminishing frequency and severity. The last one occurred at 3:00 p.m., after which the pig regained consciousness and became reoriented rapidly. By 9:00 p.m., it was eating and drinking and appeared normal.

Pig 13 was killed for necropsy on day 11. Chemical analyses of the blood of the pigs are shown (table 4).

*Necropsy Findings.*—In pig 9, dehydration was severe. The feces in the large bowel were dry and adherent to the mucosa.

*Microscopic Examination.*—The changes in the cerebral hemispheres were characterized by extensive vacuolization suggesting severe edema. The perivascular and pericellular spaces were distended (fig. 19). Many of the neurones were shrunken and hyperchromatic. Eosinophils were present around the blood vessels and in the meninges.

The mucosa of the ileum in pig 12 showed two reddened areas consisting of innumerable petechiae which became confluent centrally.

There was an abundance of eosinophils in the meninges overlying, and the perivascular spaces in, the cerebral cortex. The neurones in many parts of the cerebral hemispheres showed degenerative changes.

No significant gross lesions were observed in either pig 8 or 13.

Eosinophils were numerous in the meninges and the perivascular spaces of the

**TABLE 3—Daily Diet of 10 Pigs in Experiment 6**

| Time from start of exper. (days) | Time of day | Pen No. | Amount of swill * (kg.) | Additional water (liters) | Remarks |
|---|---|---|---|---|---|
| 1 | 10 a.m. | 4 | 8.7 | ..... | |
| | | 3 | 8.7 | ad libitum | |
| | 3 p.m. | 4 | 6.2 | ..... | ..... |
| | | 3 | 6.2 | ad libitum | |
| 2 | 8 a.m. | 4 | 8.2 | ..... | |
| | | 3 | 8.2 | ad libitum | |
| | 8 p.m. | 4 | .... | 2.5 | Pigs reluctant to eat salty food. |
| 3 | 8 a.m. | 4 | 8.2 | ..... | |
| | | 3 | 8.2 | ad libitum | |
| | 6 p.m. | 4 | 12.3 | ..... | |
| | | 3 | 16.4 | ad libitum | |
| | 9 p.m. | 4 | ..... | ..... | Pig 9 wandered around aimlessly; bumped into objects. |
| 4 | 10 a.m. | 4 | ..... | ..... | Pigs 8 and 9 showing illness; pig 6 killed; pig 9 dead after several epileptiform seizures. |
| | 1 p.m. | 4 | 11.2 | ..... | |
| | | 3 | 11.2 | ad libitum | |
| | 6 p.m. | 4 | ..... | 3.0 | |
| 5 | 9 a.m. | 4 | 5.1 | 4.0 | Pigs ate fitfully and reluctantly only after additional water given; 3 remaining pigs in pen 4 blind—2 showed involuntary jerking of the head. |
| | | 3 | 10.2 | ad libitum | |
| | 7 p.m. | 4 | 5.1 | ..... | |
| | | 3 | 5.1 | ad libitum | |
| 6 | 10 a.m. | 3 | 10.2 | ..... | Additional water withdrawn; salt feed replaced by regular feed and water. |
| | | 4 | ..... | ..... | |
| 7 | 8 a.m. | 3 | 12.3 | ..... | ..... |
| 8 | 8 a.m. | 3 | 12.0 | ..... | ..... |
| 9 | 10 a.m. | 3 | ..... | ad libitum | Pigs appeared normal; salt feed replaced by basal ration B, bran, and water ad libitum. |
| 10 | 8 a.m. | 3 | ..... | ad libitum | Pigs 12 and 13 showing characteristic seizures; pig 12 died. |
| | 2 p.m. | 3 | ..... | ..... | |
| 11 | 10 a.m. | 3 | ..... | ..... | Pig 13 still having seizures, diminishing in frequency; pig 13 appeared normal. |
| | 7 p.m. | 3 | ..... | ..... | |

* Formula of swill: 2.0 per cent NaCl, 24.5 per cent basal ration B, and 73 per cent $H_2O$.

cerebrum. Microglia and capillaries were also present in increased numbers.

*Discussion.*—The rapid onset of signs and early death of pig 9 indicate a peracute case of sodium chloride poisoning. The fact that the brain of this animal was extremely edematous lends support to the view that edema is one of the early changes, if not the first, in the brain. The clinical picture and the changes in the brain of this animal were almost identical to those seen in pig 4 (experiment 5).

According to their appearance and sodium and chloride serum levels, the pigs in pen 3 remained normal while they were given additional water, in spite of the fact that they actually consumed over 10 kg. more of the salt swill than the pigs in pen 4. Water appears to have a prophylactic effect and should always be available to pigs, particularly where garbage or other substances of doubtful composition are being fed.

The fact that pigs 12 and 13 developed the disease when reduced to the same regimen as those in pen 4 is ample proof that they were suitable controls during the first part of the experiment. It also refutes the claim made by some workers that salt starvation is necessary before sodium chloride poisoning can be produced. Why clinical signs should commence on the tenth day, 22 hours after the high salt feed was removed and an unlimited supply of fresh water given, is difficult to explain.

The high atmospheric temperature was a factor in this experiment and may have assisted materially in producing the condition on a relatively low salt intake.

## EXPERIMENT 7

This experiment was planned to estab-

**TABLE 4—Analytical Data on Serums of 10 Pigs in Experiment 6**

| Days from start of experiment | Pig (No.) | Blood serum Na (mEq./liter) | Cl (mEq./liter) | K (mEq./liter) | Ca (mEq./liter) | Remarks |
|---|---|---|---|---|---|---|
| 0 | 5 | 141 | 110 | 5.6 | 5.65 | ..... |
| | 6 | 136 | 111 | 5.0 | 5.00 | ..... |
| | 7 | 141 | 107 | 4.9 | ... | ..... |
| | 8 | 149 | 111 | 6.0 | 5.10 | ..... |
| | 9 | 143 | 107 | 4.6 | 4.00 | ..... |
| | 10 | 150 | 112 | 5.2 | 5.55 | ..... |
| | 11 | 142 | 107 | 5.3 | 5.10 | ..... |
| | 12 | 153 | 107 | 4.0 | 6.00 | ..... |
| | 13 | 133 | 113 | 5.6 | ... | ..... |
| | 14 | 162 | 115 | 5.4 | ... | ..... |
| 2 | 5 | 152 | 127 | 5.5 | 5.85 | ..... |
| | 6 | 147 | 129 | 6.1 | 4.80 | Water intake restricted until day 6. |
| | 7 | ..... | 125 | .... | ... | |
| | 8 | 134 | 121 | 4.9 | 5.30 | |
| | 9 | 154 | 125 | 4.8 | 6.50 | |
| 3 | 5 | 148 | 129 | 5.8 | 5.65 | ..... |
| | 6 | ..... | 127 | .... | ... | ..... |
| | 7 | 147 | 127 | 6.0 | 5.3 | ..... |
| | 8 | 160 | 127 | 6.9 | 6.5 | ..... |
| | 9 | 156 | 145 | 4.7 | 6.1 | Semiconscious, blind. |
| 4 | 8 | 211 | 143 | 7 | ... | Blind |
| | 9 | 209 | 147 | 4.7 | ... | Epileptiform crises; died. |
| 6 | 5 | 185 | 136 | 5.8 | ... | Listless, impaired vision. |
| | 7 | 191 | 143 | 4.7 | ... | Given fresh water and feed. |
| | 8 | 179 | 140 | 5.3 | ... | |
| | 10 | 138 | 115 | 5.7 | 5.9 | After blood samples were taken, water intake was restricted. |
| | 11 | 149 | 117 | 5.0 | 4.3 | |
| | 12 | 151 | 112 | 6.0 | 6.5 | |
| | 13 | 146 | 105 | 4.8 | 5.4 | |
| | 14 | 145 | 113 | 5.7 | 4.5 | |
| 7 | 10 | 152 | 120 | 4.6 | 5.8 | ..... |
| | 11 | 145 | 117 | 4.7 | 5.4 | ..... |
| | 12 | 143 | 127 | 6.2 | 6.7 | ..... |
| | 13 | 154 | 117 | 5.8 | 5.9 | ..... |
| | 14 | ... | 118 | ... | ... | ..... |
| 9 | 10 | 175 | 138 | 2.9 | ... | Blood samples taken before fresh water and feed were supplied at 10:00 a.m. |
| | 11 | 167 | 136 | 3.9 | ... | |
| | 12 | 176 | 139 | 5.5 | ... | |
| | 13 | 195 | 138 | 5.3 | ... | |
| | 14 | 209 | 146 | 7.5 | ... | |
| 10 | 12 | 165 | 129 | 5.9 | ... | Both pigs showing signs of illness. |
| | 13 | 180 | 125 | 5.3 | ... | |

lish whether the typical syndrome of salt or brine poisoning could be produced by a single dose of NaCl solution in distilled water given orally and, if so, what dose would be required.

*Materials and Methods.*—Eleven Yorkshire pigs, 14 weeks old, and 1, 8 weeks old, which had been fed on basic ration B and bran, were used.

At 9:00 a.m. on day 1, they were given swill which consisted of water (10 liters), basic ration B (4.5 kg.), and bran (0.3 kg.). At 10:00 a.m. they were weighed, numbered from 15 to 26, and given, by stomach tube, graded doses of 20 per cent NaCl in distilled water (table 5). The amounts given were graded, in an attempt to bracket the minimum lethal dose and the dose that would produce convulsions. The pigs were placed together in a pen where they could keep cool in mud but which contained no water for drinking. At 5:00 p.m. they were again given the same amount of swill.

At 10:00 a.m. on day 2, blood for analytical studies was taken from each animal. They were fed swill as before. (At this time 4 pigs had died.) At 5.00 p.m., the remaining pigs were given swill composed of water (6 liters), milk (4 liters), basic ration B (4.6 kg.), and bran (0.5 kg.).

On day 3, at 9:00 a.m. and 5:00 p.m., they were given swill, equal to that fed the previous night. At 11:00 a.m. 6 liters and at 2:00 p.m. 10 liters of additional water was given.

On day 4, a sample of blood was taken from pig 18 for analysis.

A routine necropsy was conducted on all pigs that died. The NaCl content of the gastric ingesta was determined in pigs 24, 25, and 26.

*Results.*—Six of the 12 pigs died in nine to 52 hours after being given the salt solution (table 5). They showed incoordination, muscular tremors, prostration with running movements, and coma before death. Pig 15 showed periodic seizures and forced circling.

The maximum NaCl level in the gastric contents of these pigs at the time of necropsy was 0.44 per cent in pig 25.

None of the other 6 pigs showed any ill effects from the saline solution during the first day. They ate the swill hungrily at 5:00 p.m. on day 2.

Pig 18 was first noticed to be abnormal at 9:00 a.m. on day 3. It was blind, pushed against objects, was incoordinate, circled, and experienced epileptiform seizures until it died at midnight on day 4.

After drinking at 2:00 p.m. on day 3, the snout of pig 21 began to twitch. The head was jerked directly back and the rump was jerked down and forward rhythmically. Following this, the pig walked aimlessly with its nose twitching and head jerking for 15 minutes. It gradually recovered and remained normal.

**TABLE 5—Weights of Pigs and Amounts of 20 per cent NaCl Solution Given by Stomach Tube (Experiment 7)**

| Pig (No.) | Amt. 20% NaCl (ml.) | Amt. NaCl (Gm.) | Wt. of pigs (kg.) | Amt. NaCl per kg. body wt. (Gm.) | Remarks |
|---|---|---|---|---|---|
| 25 | 760 | 152 | 24.5 | 6.2 | Death in 9 hr. |
| 26 | 600 | 120 | 26.4 | 4.5 | Death within 18 hr. |
| 24 | 440 | 88 | 26.6 | 3.3 | Death in 28 hr. |
| 15 | 448 | 90 | 29.1 | 3.1 | Death in 29 hr. |
| 16 | 498 | 99.6 | 37.7 | 2.6 | Death within 45 hr. |
| 17 | 456 | 91 | 34.5 | 2.6 | Death in 52 hr. |
| 18 | 425 | 85 | 38.6 | 2.2 | Signs typical of NaCl poisoning; death in 84 hr. |
| 19 | 310 | 62 | 28.2 | 2.2 | Normal. |
| 20 | 284 | 57 | 32.3 | 1.8 | Normal. |
| 21 | 324 | 65 | 36.8 | 1.8 | Mild neuromuscular signs. |
| 22 | 111 | 22 | 16.8 | 1.3 | Normal. |
| 23 | 210 | 42 | 31.8 | 1.3 | Normal. |

Pigs 19, 20, 22, and 23 showed no signs of illness. The significant increase in sodium and chloride and a slight decrease in calcium levels in the serums of the pigs in this experiment were quite constant.

*Necropsy Findings.*—No significant changes were apparent in pig 18. The mucous membrane of the stomach and intestine was normal. The necropsy findings in general consisted of varying degrees of gastroenteritis. Numerous infarcts were present in the kidneys of pigs 16 and 17.

*Microscopic Examination.*—Sections from the cerebral cortices of all pigs showed nonspecific degenerative changes with some vacuolation. Eosinophils had infiltrated the cerebral perivascular spaces in pig 17 only.

The blood vessels in the cerebrum were surrounded by many eosinophils and some round cells. Many neurons were pyknotic and shrunken. Edema, most prominent in the multiform layer of the cerebral cortex, was severe.

*Discussion.*—The course of the disease in pigs 25, 26, and 24, which were given the most salt (6.2, 4.5, and 3.3 Gm./kg., respectively) was peracute, probably the result of circulatory collapse.[41] It would appear that, because of the large dose and

the limited water intake, death occurred before the classical clinical and pathological pictures of salt poisoning had time to develop. The levels of sodium and chloride in the serum were high in these animals within three to four hours after dosing. It would be unusual for a pig to consume, voluntarily, the amounts of salt given and hence poisoning, as it occurs under practical conditions, was not observed.

Pigs 15, 16, and 17 were given less salt (table 5). The periodic attacks, circling, and unconsciousness observed in pig 15 were like those observed in pigs that had consumed an excess of salt. Litter mates 16 and 17 were the same weight and were given the same dose of NaCl under identical conditions. Pig 16 died between 36 and 46 hours, and pig 17 in 52 hours, after being given the salt solution. Eosinophils were observed only in the brain of pig 17. It is tempting to postulate that more than 36 hours is necessary after the ingestion of sodium chloride for the conditions responsible for the infiltration of eosinophils into the brain to develop. The infarction of the kidneys in these animals was probably the result of vascular spasm, as no organic basis for them could be established.

Both the clinical and pathological pictures in pig 18 (given 2.2 Gm./kg. of NaCl) were typical of those exhibited by the pigs fed NaCl over a period of time.

## Experiment 8

Since we were able to produce the characteristic syndrome and pathological changes by feeding NaCl, and as it is known that the two ions dissociate readily in the animal body, it was suggested that only one of the two was responsible.

Experiment 8 was set up to determine which of the ions (sodium or chloride) was the etiological agent.

*Materials and Methods.*—Eight litter mate Yorkshire pigs, 3 months old, were allotted to two groups of 4. They were weighed and placed in separate pens.

Group 1 was fed swill consisting of sodium propionate (330 Gm.), basic ration B (2 kg.), and water (6 liters). The concentration of sodium propionate, 330 Gm., or 4.0 per cent, is equivalent in sodium to 200 Gm. or 2.5 per cent NaCl.

Group 2 was given swill containing potassium chloride (130 Gm.), calcium chloride (95 Gm.), basic ration B (2 kg.), and water (6 liters). The concentration of potassium chloride, 130 Gm. or 1.58 per cent, and of calcium chloride, 95 Gm. or 1.16 per cent, are essentially equivalent in chloride to 200 Gm. or 2.5 per cent NaCl.

Additional milk and water were added to the swill of both groups when it was obvious that the pigs were not going to eat it otherwise.

All the pigs were weighed and blood samples were taken from those in group 1 on day 4. The feed containing sodium propionate and potassium and calcium chloride was removed at 5:00 p.m. on day 5, and regular feed and water were given.

A blood sample was again taken from pig 43 on day 6 and chloride, sodium, and potassium levels in the serum were determined. It was killed for necropsy at 11:00 p.m. on day 7.

*Results.*—The pigs in group 2 ate their 10.25 kg. of swill reluctantly, so at noon, 1 liter of water and 1 liter of milk were mixed with the swill. The remainder was eaten during day 2 after an additional 3 liters of water and 1 kg. of pig feed were added. The 8.5 kg. of swill given on day 2 was diluted with a total of 6 liters of milk and 2 kg. of basic ration B on day 3. However, 2 kg. of the mixture still remained in the trough when it was discarded and fresh feed and water were given on day 5. These pigs became severely dehydrated, were listless, and walked with their backs arched. They lost an average of 15 per cent of their original weight. However, no other signs were apparent. When given the regular feed and water, they ate and drank greedily. Within 24 hours, they had regained their normal vigor and appearance.

The pigs in group 1 ate the swill containing sodium propionate readily during the first two days but ate little thereafter. They consumed 16.6 kg. on day 1, about 12 kg. on day 2, and only small amounts on days 3 to 5. During the last three days, a total of 6 liters of milk and 2 kg. of hog grower were used to dilute the swill (not more than 4 kg.) remaining in the trough after the second day. Even then, about 2 kg. of the diluted mixture was thrown away so that fresh feed and water could be given at 5:00 p.m. on day 5.

All 4 pigs consumed the fresh food and water readily, and all appeared normal. They had lost an average of 10 per cent of their original weight; not as much, nor did they appear as dehydrated, as pigs in group 2.

On the sixth day, pig 43 (sodium group) did not come to the trough as did the other pigs at the 9:00 a.m. feeding (16 hours after the sodium feed was removed). It was found to be unaware of its surroundings, was apparently blind, did not respond to

external stimuli, and wandered around aimlessly.

At 9:30 a.m. its snout began to twitch periodically. At 10:00 a.m., the nose twitch increased in frequency, then clonus of the neck muscles commenced. The head was jerked directly backward. Forced circling, within a very narrow radius, while the snout was lowered to, and placed on, the ground characterized some of the seizures. The pig pushed forward as it circled, the snout remaining fast on the ground until suddenly the neck bent, allowing the forehead to contact the ground. Equilibrium was then lost and the pig tumbled over. It fell on its side with limbs rigidly extended while respiration was suspended and cyanosis developed; the pig then relaxed and respiration was resumed.

Similar attacks, except for the somersault, occurred at 10:30, 10:37, and 10:45 a.m. Between attacks, the pig lay relaxed or got up and wandered aimlessly. Upon arriving at a corner, it frequently pushed its head up and raised its front legs alternately as though it were trying to climb.

Fits continued to occur at irregular intervals throughout the day. Forced circling, pleurothotonos, and pivoting with the snout pressed to the ground characterized most of the attacks.

It was killed at 11:00 p.m. The other pigs remained normal.

*Necropsy Findings.*—No significant gross changes were present.

*Microscopic Examination.*—Sections of the cerebral cortex showed changes characteristic of those seen in the pigs poisoned with NaCl. Many neurones were shrunken, angular, and pyknotic. Moderate edema was evidenced by distention of pericellular and perivascular spaces. Numerous eosinophils surrounded the blood vessels and had infiltrated the pia mater (fig. 20).

*Discussion.*—The clinical and pathological picture produced in pig 43 by feeding sodium propionate was the same as that observed in the pigs fed NaCl. This would suggest that the sodium rather than the chloride ion is responsible for the disease.

Simple dehydration, since it was much more severe in group 2 than in group 1, does not appear to be the important factor in the pathogenesis of the disease.

It is of interest that signs were first noticed in pig 43, 16 hours after it had been given, and apparently had consumed, feed and water without additional salt. The same phenomenon was observed in pigs 12 and 13 in experiment 6.

EXPERIMENT 9

A series of experiments was conducted to determine whether the classical clinical and pathological changes of salt poisoning could be produced by simply raising the serum levels of sodium and chloride to or above the levels previously observed. Varying amounts of 20 per cent NaCl solution were given as a single intravenous injection to 12 pigs. Of these pigs, 3 died within 15 minutes, 1 in 12 hours, and 1 in 24 hours. Intracranial hemorrhage and blanched areas in the kidneys were revealed at necropsy. None showed the clinical or pathological manifestations which we had come to consider typical of salt poisoning. The rest of the pigs showed no ill effects.

*Discussion.*—At least transitory concentrations of sodium and chloride were attained in the serums of these pigs equal to or in excess of those seen in pigs poisoned by oral consumption of NaCl. However, the clinical and pathological pictures did not correspond to those seen when pigs were fed the salt. It would appear, therefore, that there is a difference in the mechanism by which these two methods of administration produce disease in the pigs. The intravenous administration of such large doses of salt probably produces acute salt poisoning by dehydration of the cells and simultaneous disturbance of ionic equilibrium, as postulated by Volker.[41] Concomitant increase of intravascular pressures and systemic circulatory disturbances are indicated by the intracranial hemorrhagic and blanched areas in the kidneys of these pigs. The signs observed suggest that the effects of massive transfusion with NaCl are produced immediately and that, if death or fatal injury does not occur at once, the animal recovers. On the other hand, to produce the characteristic clinical and pathological pictures observed after oral administration of the NaCl, a period of up to six days is required during which a moderately high level of NaCl has probably been maintained in the digestive tract with continuous absorption into the blood serum. This continued high level is perhaps necessary in order that the sodium or chloride, or both, may become sufficiently concentrated in the brain tissue to bring

about the changes and the signs which we associate with typical salt poisoning.

### EXPERIMENT 10

A study of the reaction of brain tissue to injection of NaCl into the cerebral hemispheres was considered as a probable approach to the understanding of the lesions in the brain in salt poisoning.

It was decided to determine if the NaCl or its ions, alone or mixed with damaged brain tissue, could be responsible for the characteristic eosinophilic infiltration.

*Material and Methods.*—Four Yorkshire pigs, 4 months old, were used. Pig 48 was given 1.0 ml. of 4 per cent NaCl solution intracerebrally and killed on the second day. Pig 49 was injected with 0.5 ml. of 20 per cent NaCl in each cerebral hemisphere and, on the fourth day, it was killed. Pig 50 received 0.5 ml. of a saturated solution of NaCl in each cerebral hemisphere and was killed on the fourth day. Pig 51 was given 0.7 ml. of saturated saline solution in the left cerebral hemisphere. Seven days later, when a second injection was attempted, the animal died under anesthesia.

All the pigs were killed by electricity and exsanguination. The brains were fixed and sectioned. All the surgical procedures were carried out under deep anesthesia and aseptic technique.

*Results.*—Pig 49 appeared partially blind and tilted its head to the left after the operation. Pig 51 became cyanotic immediately after the injection but recovered and remained clinically normal until euthanasia. The other 2 pigs remained normal.

*Necropsy Findings.*—The trephine holes in the skull and the incision into the skin were clean and healing in each pig. The site of injection in the cerebrum was readily demonstrated by hemorrhage and blood pigment in pigs 49, 50 and 51.

*Microscopic Examination.*—There was no reaction around the needle punctures in the cerebrum of pig 48. A break in the tissue and a few groups of erythrocytes were the only evidence of damage. Hemorrhage had occurred into the injection site in pig 49. A few neutrophils were present and round cells had infiltrated. No reaction was apparent in the surrounding tissue. Hemorrhage with malacia had developed around the site of injection in pig 50. A few neutrophils and round cells had infiltrated the pink amorphous granular zone extending out from the puncture wound.

The cerebrum of pig 51, which had been kept alive for seven days following injection of a saturated solution of NaCl, showed blood pigment, a few neutrophils, round cells, and an occasional eosinophil in and around the injection. The nervous tissue in the cerebral cortex adjacent to the injury showed increased cellularity. Microglia had developed into compound granular corpuscles and were filled with fatty material. Endothelial cells were swollen and capillaries had proliferated. No eosinophils were associated with the blood vessels.

*Discussion.*—The results of this experiment show that the mere presence of NaCl and damaged nerve tissue within the brain does not excite an eosinophilic reaction comparable to that observed in NaCl poisoning. The capillary proliferation and microglial reaction are not specific.

### EXPERIMENT 11

The fact that eosinophils characterized the early changes in the brain in salt poisoning led us to the hypothesis that the disease was really brought about by an allergic or sensitivity reaction. A search of the literature failed to reveal an account of the effects of iso-allergic encephalomyelitis in swine.

Two Yorkshire pigs, 2 months old, were injected in the axial region with pig brain and adjuvants. Four weeks later intracerebral administration of emulsified pig brain in normal saline solution resulted in incoordination and paralysis terminating in death. The microscopic changes in the brain were characterized by demyelineation, infiltration of lymphocytes, and large round cells. A few neutrophils were present but eosinophils were rarely seen.

*Discussion.*—The results of this experiment indicate that both the clinical and pathological picture of iso-allergic encephalitis in the pig are readily differentiated from NaCl poisoning of swine.

## DISCUSSION—GENERAL

The results of these studies show that the clinical syndrome associated with the feeding of brine or salted fishmeal to swine can be produced by simply adding pure NaCl to the regular ration of pigs and giving a limited but variable amount of water. The toxins, thought by many to play such an important role in brine and fishmeal poisoning, may have been present in some instances but these experiments leave little

doubt that NaCl, probably the sodium ion, was the principal etiological agent. The role of water is important since the condition did not occur when an unlimited supply of fresh water was given. However, by restricting the water supply the disease was produced by the addition of as little as 2 per cent of NaCl to the regular ration (total NaCl 2.5%). The fact that sodium propionate experimentally produced the typical clinical and pathological syndrome suggests that the sodium ion is responsible.

The clinical picture of deranged consciousness, blindness, and the epileptiform seizures beginning with twitching of the nose and progressing to clonus of neck muscles, circling or running movements, retropulsion, pleurothotonos, opisthotonos, and sialorrhea, which characterize the typical case of NaCl poisoning appears to be unique in this disease. Enterotoxemia (gut adema) of pigs may resemble certain features of NaCl poisoning. In typical salt poisoning, however, the animal is unconscious and hence does not respond to external stimuli, whereas a response can be elicited and is often accentuated in an enterotoxemic pig.

The results of the experiments indicate that the microscopic changes in the cerebral cortex, consisting of edema and eosinophilic meningoencephalitis with malacia are pathognomonic. Both the sodium and chloride levels of the blood were significantly elevated in nearly all our experimental animals. This finding may be of value in making a differential diagnosis. Chemical analyses of the stomach contents did not prove to be reliable diagnostic aids. Apparently NaCl is absorbed rapidly from the gastrointestinal tract.

The pathogenesis of salt poisoning remains in doubt. Several facts revealed during the experiments must be considered of importance:

1) The characteristic syndrome of classical salt poisoning was not produced by intravenous administration of a single dose of NaCl solution.

2) It was necessary to feed salt for at least 39 hours (exper. 5, pig 4) and up to seven days (exper. 2, pig 8) before the clinical and pathological pictures typical of NaCl poisoning in swine could be produced.

3) The disease did not occur when fresh water was available continuously.

4) When the water was restricted severely at first, and then given at intervals, the disease appeared to occur more readily (exper. 5).

5) In several instances, the disease occurred after the high salt feed had been taken away and the pigs given adequate fresh water and feed (exper. 6, pigs 12, and 13; exper. 8, pig 43).

It appeared to be necessary to raise and keep the concentration of salt in the blood stream of the pig high for a time. When an unlimited supply of water was available, large amounts of salt were eliminated rapidly (exper. 4, pig c). A high concentration of sodium was maintained in the blood by giving large amounts in the feed (exper. 2, 3, and 5) with moderate restriction of water intake, or by restricting the water intake severely in the presence of a lesser increase in NaCl (exper. 6).

It has been shown by Behrens [2] that in NaCl poisoning, local osmotic disturbances and gastroenteric changes have no importance, but signs of toxicosis and death after oral intake are the result of changes which occur after the salt has been taken into the blood. Therefore, attention should be given to pertinent information regarding the action and fate of NaCl in the blood.

Cannon [6] attempted to explain the progessive increase in intracranial pressure following trauma on the basis of edema of the injured brain causing pressure on the blood vessels and further edema leading ultimately to anemia of the brain from vascular compression. He postulated that brain tissue deprived of blood underwent chemical changes resulting in greater osmotic pressure and further passage of water into the tissues. He found that brains placed in normal saline solution increased continuously in weight up to 33 per cent. He stated that, "The main force in causing swelling is probably osmotic pressure which, in brain tissue, may attain a degree so much greater than blood pressure that the blood would really be prevented from entering the cranium." He accounted for recovery from brain injury on the basis of diffusion of the products of destruction from the tissues.

The results of experiments by Weed and McKibben [42] suggested that a reduction in the volume of the brain followed intravenous administration of hypertonic saline or glucose solutions, while hypotonic solutions caused an increase in the intracranial pressure. The results of Cushing and Foley [8] and of Foley and Putnam [10] were interpreted as confirmation of the general conclusions of Weed and McKibben. They also demonstrated that if an animal had

been given a concentrated saline solution the day before, the rise in pressure following the administration of water (a hypotonic solution) was more marked and of longer duration.

As a result of the foregoing experiments, NaCl was recommended and used to reduce the increased intracerebral pressure following trauma. However, Hoff[15] described a series of experiments in which he demonstrated that the brain tissue gave up some of its fluid content for a brief period while the salt was still present in the blood stream, but later, when the salt had passed from the blood stream into the brain cells, "which seem to have an avidity for sodium chloride," fluid flowed back into the cells and the swelling of the brain recurred to a greater extent than before the treatment.

Browder issued a warning, based largely on clinical observations, against the use of hypertonic solutions when the brain bulk was increased. Milles and Hurwitz,[25] using improved apparatus, observed a secondary rise in pressure which reached a point much higher than the original. They attributed this phenomenon to the fact that the NaCl passed from the blood to the tissues. Subsequent elimination of the solute via the kidneys rendered the blood hypotonic to the issue, thus a reversal of the original situation occurred and fluid passed back into the tissues.

Jackson *et al.*[16] also observed a secondary rise, more marked with NaCl than with glucose, which they attributed to an accumulation of the salt in the brain cells. They suggested that prolonged pressure on the cortex of the brain may result which, in turn, may lead to atrophy of the cortical nerve cells by restriction of cerebrospinal fluid and blood flow.

Masserman[21] suggested that the reduction in cerebrospinal fluid pressure after administration of hypertonic solution was due both to a resorption of spinal fluid and to a diminution in the volume of the nervous tissue itself. Conversely, the rise in cerebrospinal fluid pressure to normal and higher, subsequent to the period of depression would, theoretically, be due to the fact that the blood, through the loss of its solutes in the urine, had meanwhile been rendered hypotonic to the nervous tissue. The reason given by Masserman for the increase in pressure to above preinjection level was that, while their levels in the blood were high, dextrose or NaCl was deposited in the nervous tissues; thus, compensatory edema resulted when the blood level of the solute was subsequently reduced.

Bulloch *et al.*[5] compared the effects on the intracranial pressure of intravenous injection of glucose and NaCl. The pressure was reduced for two to three hours, then rose above normal, exceeding it by 40 mm. of Ringer's solution in the case of glucose and 110 mm. when NaCl was used.

Wilson *et al.*,[43] employing more dilute solutions of NaCl than previous workers, did not observe the secondary rise in pressure in anesthetized dogs.

In summary, it would appear that, except for those of very low hypertonicity, hypertonic solutions of dextrose and NaCl initially reduce, but secondarily increase, the intracranial pressure; the secondary increase being due to diffusion of the solute into the brain tissue and subsequent imbibition of fluid when the blood, through renal excretion, becomes hypotonic to the tissue.

In our experiments, the amount of NaCl consumed by the pigs was large and the water intake was reduced. Thus, the level of sodium chloride in the blood, lacking the vehicle in which it could be eliminated, rose and remained high. It is reasonable to assume that, by the process of impeded diffusion, the salt or its ions passed the "blood brain barrier" and became concentrated gradually but progressively in the nervous tissue.[29] By renal excretion, the concentration of sodium and chloride was reduced in the blood. A quantity of water given at this time further diluted the blood. There was a lag period in the transfer of ions from the brain back to the blood similar to that which occurred when they entered the brain. The net result under these conditions could conceivably result in massive edema of the brain. The fact that the signs were not manifested by the experimental pigs, in several instances, until after fresh water in abundance was made available, and that severe edema was observed microscopically in the cerebrums of pigs that died shortly after the onset of signs, tends to support such a proposition.

The focal nature and location of the lesions are difficult to explain. It could be that the edema is more severe and destroys tissue in that part of the cerebral cortex forming the walls of the sulci. This would

presuppose a concentration of the sodium in these areas. Certainly, the cortical tissue, except for the molecular layer which was less severely injured, is a highly specialized tissue and hence more sensitive to injury. The suggestions of Cannon [6] and Jackson *et al.*[16] regarding increased intracranial pressure leading to edema, anemia, and injury, although highly speculative, may have some application here. It would be of interest if one could determine whether the focal areas of malacia observed were the parts of the cerebral cortex most likely to be deprived of their blood supply if pressure did reduce blood flow. The neurones in the areas involved are highly developed and active, which would render them more susceptible to injury through lack of oxygen. As pointed out by McFarland,[22] cortical tissue is permanently damaged if it is deprived of oxygen for more than five to ten minutes while, in other parts of the central nervous system, irreversible changes do not begin to occur for periods of up to 30 minutes.

Utter [39] has proved that sodium is a strong inhibitor of anaerobic glycolysis in brain through stimulation of conversion of adenosinetriphosphate to adenosinemonophosphate (AMP) and a decreased rate of removal of AMP by phosphorylation. The resulting accumulation of AMP inhibits glycolysis. The combination of reduced oxygen supply from increased intracranial pressure and inhibition of anaerobic glycolysis may account for the damage to the brain in NaCl poisoning. Fluoride is also an inhibitor of glycolysis.[12] If this inhibitory effect is a factor, then sodium fluoride should be superior to NaCl in producing salt poisoning. An additional consideration is the reduction of food intake by pigs after a few days on high salt feed, which would tend to lower the blood and, secondarily, the brain sugar level.

The almost exclusively eosinophilic infiltration is not explained on this basis since, in experiment 10, the brain was damaged in the presence of NaCl without eosinophils appearing in significant numbers. Such selective attraction of eosinophils outside of allergic and sensitivity reactions is almost unique in sodium salt poisoning and may be a clue to the role of these cells in the body.

Selye [34] observed that chronic treatment with desoxycorticosterone, especially after sensitization by unilateral nephrectomy and a high sodium intake, caused severe and permanent lesions in the brains of rats. In young rats, massive edema of the cerebrum was suggested as the cause of death. Edema and liquefaction necrosis characterized the lesions in the cerebral cortex. These lesions resulted in clonus of neck muscles, backward jerks of head and shoulders, and salivation. The attack was frequently terminated by the animal falling to the floor in an apparently unconscious state.

The calcium level in the serum of our experimental pigs remained normal, usually in the high normal range. It is unlikely, therefore, that calcium deficit is an important factor in salt poisoning.

Determination of the cerebrospinal fluid pressures and the sodium contents of the brains during the production and course of this disease may prove to be fruitful lines of investigation in determining the exact pathogenesis of sodium salt poisoning.

## SUMMARY AND CONCLUSIONS

The experiments and observations recorded in this paper establish sodium salt poisoning in pigs, apparently for the first time, as a clearcut, readily recognizable pathological as well as clinical entity. The results obtained from the experiments involving 53 pigs seem to warrant the following conclusions.

1) The clinical syndrome associated with the feeding of meat, fish brine, or salted fishmeal to swine can be produced by adding pure sodium chloride (NaCl) to the regular ration of pigs, and giving limited but variable amounts of water.

2) The sodium ion and a body fluid volume disturbance are reciprocal etiological factors in salt poisoning of swine.

3) The toxins thought by many to play an important role in brine and fishmeal poisoning, if present at all, are purely incidental. The NaCl content is the principal etiological agent.

4) The disease can be produced by inclusion of as low as 2.5 per cent NaCl in the swill when water intake is restricted.

5) Twenty per cent aqueous solution of NaCl, given by stomach tube to 4-month-old pigs at the rate of 2.2 Gm. per kilogram of body weight, will cause death, and 1.8 Gm. per kilogram of body weight will produce epileptiform seizures. Such values are

relative and will vary according to the amount of water made available to the pigs.

6) Sodium propionate, in a 4 per cent concentration in the feed, will cause clinical and pathological manifestations identical to those seen in the experimental pigs poisoned with NaCl.

7) Pigs can tolerate a high sodium salt intake when fresh water is available at all times.

8) It is necessary to keep the level of sodium in the blood high for a time before the syndrome and lesions of classical salt poisoning will occur.

9) The intravenous injection into pigs of single large doses of 20 per cent aqueous solution of NaCl causes major circulatory disturbances evidenced by renal infarction, intracranial hemorrhage, and death from respiratory arrest. The typical syndrome of sodium salt poisoning was not produced in this way.

10) Macroscopic lesions in animals poisoned by salt are nonspecific.

11) The microscopic changes in the cerebral cortex, consisting principally of edema and eosinophilic meningoencephalitis with malacia, are pathognomonic of poisoning by sodium salt in swine.

12) The lesions which occur in isoallergic encephalitis in pigs do not resemble those seen in sodium salt poisoning.

13) The injection of NaCl into the cerebrums of pigs does not result in infiltration of eosinophils.

14) Both the sodium and chloride levels in the blood serum in NaCl poisoning are valuable differential diagnostic aids.

15) An eosinopenia accompanies the onset of signs and persists during the course of the disease. An increase in the number of eosinophils signals recovery.

16) Blood serum calcium levels are normal in pigs suffering from excessive intake of sodium salt.

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# Strain Variation in the Response of Sheep Nematodes to Action of Phenothiazine. III. Field Observations

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THE EFFICACY of phenothiazine as an anthelmintic for gastrointestinal sheep nematodes has been established both therapeutically [6,7,11,14,17] and prophylactically.[15] Continued effectiveness of phenothiazine-salt mixtures, over extended periods, has been observed by several workers.[12,13,18] However, concern exists over the selection of drug-fast strains of nematodes from the prolonged and intensive use of this compound.[2,3,10]

Numerous reports from sheepmen of the central Kentucky region, together with information from the departmental diagnostic laboratory, suggested that phenothiazine was no longer effectively controlling the parasites of sheep. Indeed, some sheepmen have discontinued its use as a drench while others have discontinued the salt mixture. Therefore, studies were undertaken in an attempt to offer some explanation for the apparent failure of phenothiazine.[4,5]

The field studies, which were mentioned briefly in an earlier paper [4] and will be reported here in detail, were purposely withheld pending confirmation by more adequately controlled laboratory experiments.[4,5]

## MATERIALS AND METHODS

Three flocks in the central Kentucky area, representing different medicinal procedures with varying degrees of phenothiazine exposure, were selected and the parasite picture in each followed for a period of one and a half years. The flocks were composed of purebred animals and the introduction of new individuals was minimal. A description of the individual flocks and the medicinal control procedures used by each follows:

*Farm K.*—This flock contained 85 ewes (50 Southdowns, 35 Hampshires) and the medications utilized were 1–9 phenothiazine-salt mixture and drenches with copper sulfate-nicotine sulfate (Cunic). The drench was composed of 1 oz. Blackleaf 40 (40% nicotine sulfate) and 1 oz. bluestone (copper sulfate) in 3 qt. of water. Southdown ewes were given 3 oz. and the Hampshire ewes 4 oz. of the solution. These ewes raised 100 lambs (50 Southdowns, 50 Hampshires) which were drenched at the rate of approximately 1 oz. per 25 lb. of body weight. The salt mixture was available to both ewes and lambs at various periods.

*Farm S.*—Periodic drenching with phenothiazine-lead arsenate was the only parasite-control medication utilized in this flock of 75 Southdown ewes. The ewes were given 2 oz. of the suspension which delivered 25 Gm. of phenothiazine and 1 Gm. of lead arsenate. The lambs (approx. 80) were given 1 oz. of the suspension before weaning and 2 oz. thereafter.

*Farm B.*—Phenothiazine-salt (1–9 mixture) and drenches with phenothiazine-lead arsenate (same commercial suspension and dose rates as used on farm S), copper sulfate (1% sol., 1.0 to 1.5 oz. per lamb), and copper sulfate-nicotine sulfate (same solution and dose rate as on farm K) were the parasite-control medications used on this Southdown flock of 150 ewes and approximately 120 lambs. The strain B worms utilized in experiments reported in two previous papers [4,5] were originally isolated from sheep on farm B.

Ten per cent or more of the animals of each flock were initially selected and rectal feces were obtained from these animals at two-week intervals, in most cases, throughout the course of the study. Worm egg counts of the individual animals for the various groups were averaged on each collection date for presentation in the graphs (1–7) of this report.

Stoll's salt flotation method [16] for the estimation of eggs per gram (e.p.g.) of feces was modified to obtain dilution factors ranging from 10 to 100. Differential worm egg counts were made, but eggs were grouped for presentation in the present paper in the following manner: total count, total count minus Strongyloides, and total Haemonchus. The estimation of larval development was essentially the same as that recorded in an earlier paper.[4] The ratio, larvae per gram/e.p.g. (exclusive of Strongyloides), was used to express the percentage of larval development. When fecal sampling was made on extremely hot days, an ice chest was used to prevent the eggs from developing before arrival at the laboratory.

From the Department of Animal Pathology, Kentucky Agricultural Experiment Station, Lexington.

This investigation is in connection with a joint project of the Kentucky Agricultural Experiment Station and the Agricultural Research Service, U. S. Department of Agriculture. It is published by permission of the director of the Station and the chief of the Animal Disease and Parasite Research Branch, ARS.

Climatological data were obtained from the U. S. Department of Commerce Weather Bureau, Lexington, Ky., and though incorporated in graph 3 only, apply to all graphs (1 to 7).

The reliability of a field study of this type is considerably dependent on the interest, reliability, and cooperation of the flock owners and their hired help. The choice of the above farms was made, in addition to the varying phenothiazine usage, with this consideration in mind. It is the authors' opinion that the information obtained regarding dates, dosing, and availability of salt mixture is entirely reliable.

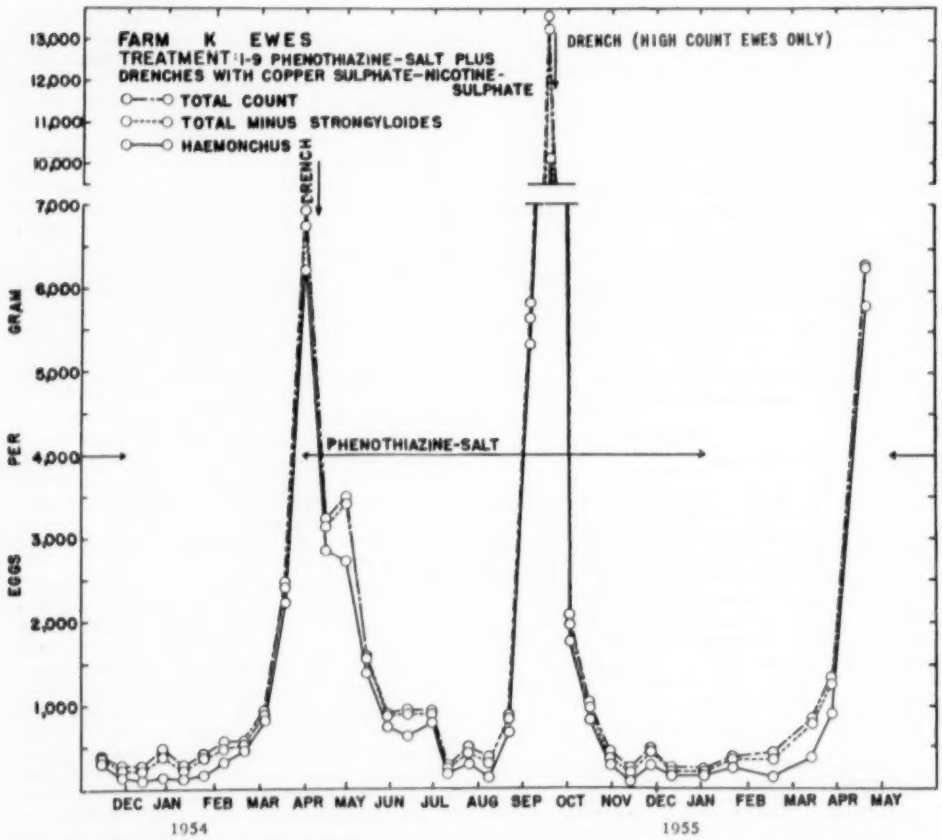
Since one of the objectives of these field trials was to assess the relative values of the different parasite control medications used on the three farms, no changes in their medication program were instituted by the authors.

## RESULTS

The worm egg counts of the individual animals of the respective flocks, were averaged for each collection date and are presented together with the pertinent medications in graphs 1 to 7.

The farm K breeding ewes (graph 1) were drenched on two occasions with Cunic. Because of a misunderstanding by the herdsman, only 3 ewes with high egg counts (30% of ewes) were drenched in September, 1954. A sharp reduction in egg counts was observed following each drench. It is of interest that the highest egg count peak occurred in this flock while the ewes had access to the phenothiazine-salt mixture.

The three phenothiazine-lead arsenate

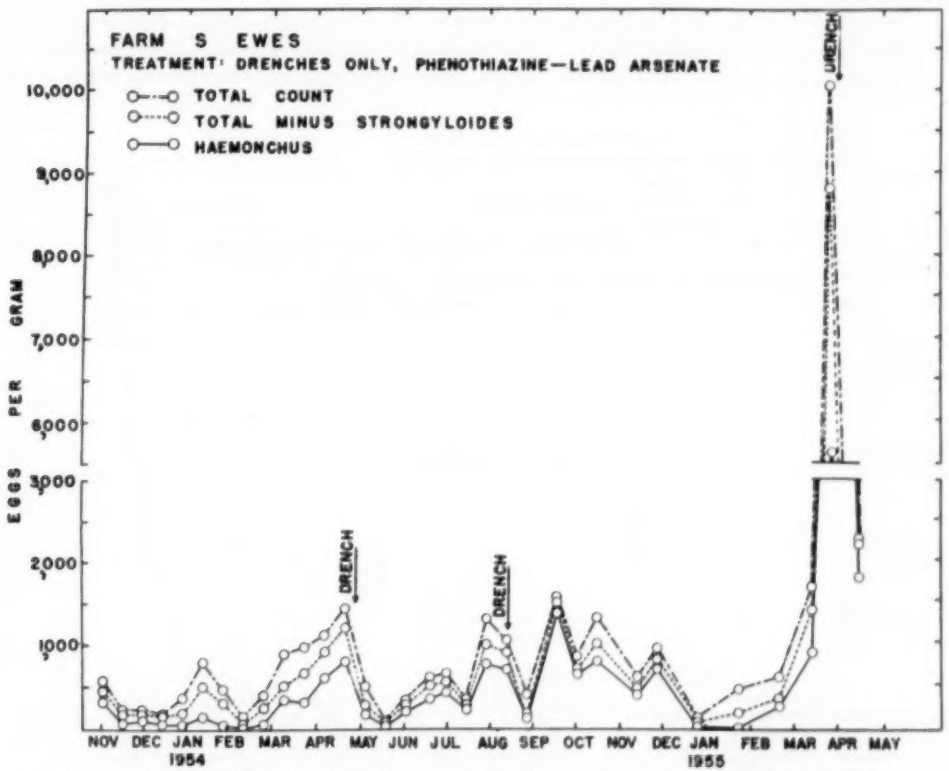



Graph 1—Effect of phenothiazine-salt mixture and copper sulfate-nicotine sulfate drenches on the average nematode egg counts (eggs per gram of feces) of farm K ewes. Egg counts were made at approximately two-week intervals.

drenches employed on farm S ewes (graph 2) appeared effective, as indicated by a subsequent reduction in the egg count. The marked difference between the 1954 and 1955 spring rises in egg counts was due, at least in part, to the drought conditions which existed in 1954. The climatological data (graph 3) show that the precipitation varied approximately from one half (Feb., 1954) to twice (Feb., 1955) the average of the preceding 54 years. This difference in spring rises was also experienced in the farm B ewes.

The breeding ewes of farm B (graph 3) were drenched four times during the observation period. The first drench (Oct., 1953) was given just prior to the first fecal collection, so comparison of the pre- and post-treatment egg counts was not possible. It is likely that the egg count for that time of year was at a low level, so little or no reduction could be expected. Following the second drench (March, 1954), no reduction in egg count occurred within the period during which the drench could be considered responsible. At the time of this second drench, the phenothiazine-salt mixture was made available. The counts at the time of the third drench were of a low order. However, the fourth drench, in April, 1955, was administered when the average Haemonchus count was 3,500 e.p.g. Following this drench, there was an increase to 7,700 e.p.g. In addition, the phenothiazine-salt mixture was available to the ewes following the fourth drenching. The difference in egg count peaks in the springs of 1954 and 1955 was similar to that in farm S ewes.

Following each of the four copper sulfate-nicotine sulfate drenches of farm K lambs (graph 4), a reduction of the aver-



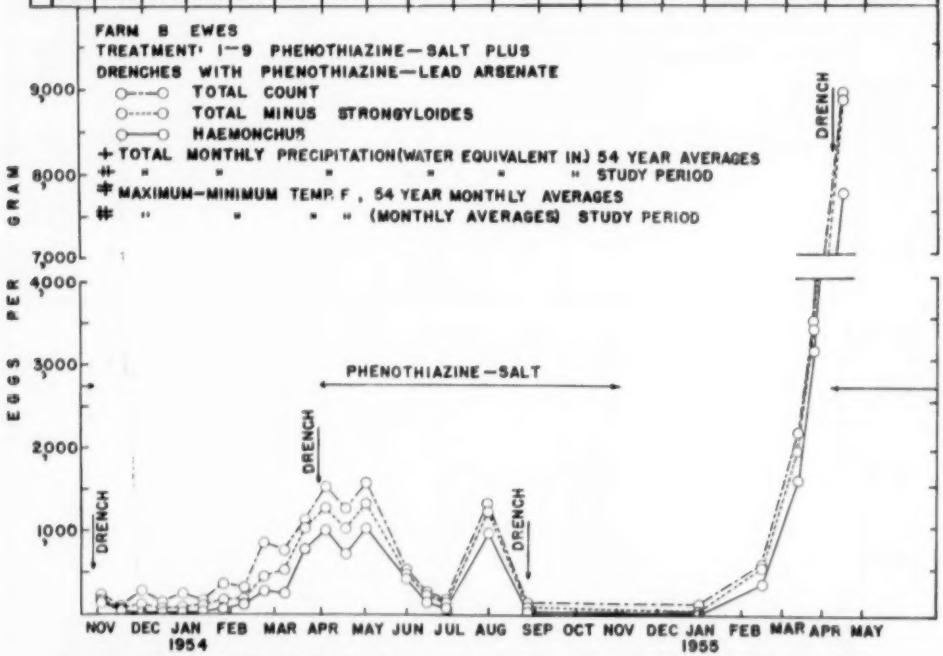

Graph 2—Effect of phenothiazine-lead arsenate drenches on the average nematode egg counts (eggs per gram of feces) of farm S ewes. Egg counts were made at approximately two-week intervals.

age egg count was observed. Although the drenches were effective, there was a tendency for the general level of parasitism to increase during this period when the phenothiazine-salt mixture was available. The egg count began to rise again in March, 1955, but it is difficult to credit this rise to the absence of the salt mixture alone, since this was also the period of increased precipitation and optimum temperatures (graph 3). A rise in the egg count was also observed in farm S lambs during this period and no salt mixture had been used.

The farm S lambs (graph 5) were drenched five times with phenothiazine-lead arsenate. Following four drenches, a noticeable reduction in egg count occurred. The egg count at the time of the April, 1954 drench was at a low level and a distinct reduction was not observed. Although the egg count continued to decrease through October and into November following the October 4 drench, it is unlikely that a single drench would have had this prolonged effect.

At farm B, it was the management practice to separate the ewe and ram lambs after weaning (July 4) and, since the treatments and dates of treatment varied with each, they are treated separately in the present discussion. The ewe lambs (graph 6) were given eight drenches (6 phenothiazine-lead arsenate, 1 copper sulfate, and 1 Cunic) and the phenothiazine-

| | OCT | NOV | DEC | JAN | FEB | MAR | APR | MAY | JUN | JUL | AUG | SEP | OCT | NOV | DEC | JAN | FEB | MAR | APR | MAY |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| + | 2.40 | 3.18 | 3.63 | 4.49 | 3.12 | 4.52 | 3.54 | 3.63 | 4.06 | 4.25 | 3.35 | 2.78 | 2.40 | 3.18 | 3.63 | 4.49 | 3.12 | 4.52 | 3.54 | 3.63 |
| ++ | 1.12 | 1.46 | 2.44 | 4.16 | 1.68 | 2.38 | 3.79 | 2.23 | 2.14 | 3.82 | 3.29 | 4.97 | 1.33 | 2.36 | 3.38 | 1.57 | 7.69 | 7.03 | 1.71 | 6.43 |
| * | 67.7 | 53.2 | 63.6 | 42.0 | 43.7 | 53.3 | 63.8 | 74.0 | 82.9 | 86.1 | 84.6 | 79.5 | 67.7 | 53.2 | 43.6 | 42.0 | 43.7 | 53.3 | 63.8 | 74.0 |
| | 47.4 | 36.0 | 28.4 | 26.2 | 26.8 | 34.7 | 44.1 | 54.1 | 63.3 | 66.6 | 65.1 | 59.0 | 47.4 | 36.0 | 28.4 | 26.2 | 26.8 | 34.7 | 44.1 | 54.1 |
| # | 75.1 | 57.7 | 44.3 | 43.7 | 54.0 | 53.5 | 72.7 | 71.1 | 87.2 | 92.2 | 86.6 | 86.4 | 68.1 | 53.1 | 42.3 | 40.2 | 44.9 | 56.5 | 70.4 | 76.8 |
| | 48.4 | 34.8 | 25.7 | 26.9 | 31.7 | 32.1 | 48.9 | 49.0 | 63.2 | 67.1 | 66.2 | 60.7 | 47.9 | 36.0 | 28.0 | 24.0 | 27.8 | 36.4 | 47.5 | 55.5 |




Graph 3—Effect of phenothiazine-lead arsenate drenches and phenothiazine-salt mixture on the average nematode egg counts (eggs per gram of feces) of farm B ewes. Egg counts were made at approximately two-week intervals. The numbers at the top of the graph indicate the variations in precipitation and temperature from October, 1953 to May, 1955; these data apply to all graphs (1-7).

**TABLE 1—Percentage* of Larval Development**

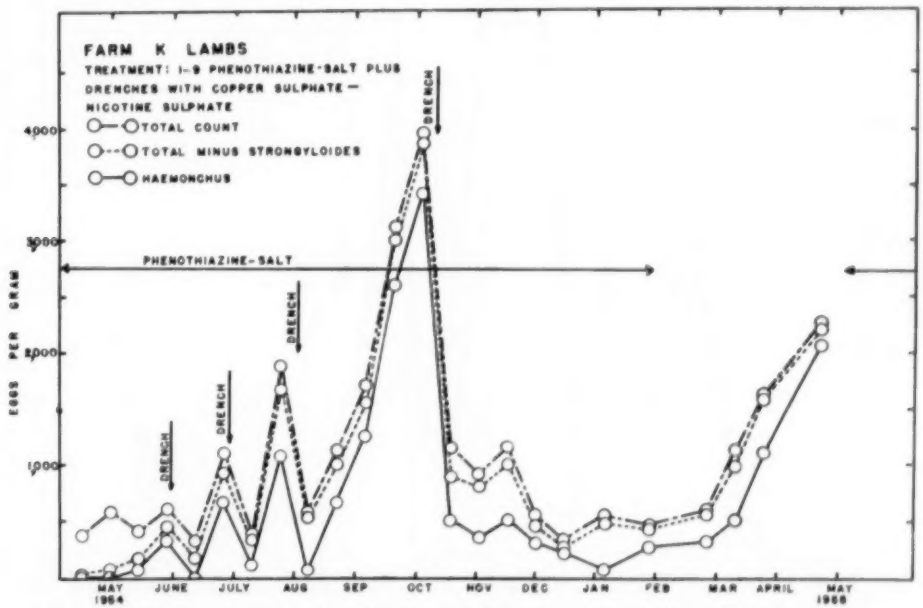
| Farm | Phenothiazine-salt mixture | Lamb averages | | | Farm average | Ewe averages | | | | Farm average |
|---|---|---|---|---|---|---|---|---|---|---|
| B | On | 29.1 | 46.8 | 74.0 | 50.0 | 69.0 | 29.5 | 34.9 | ..... | 44.5 |
| | Off | ..... | ..... | 65.8 | 65.8 | ..... | ..... | ..... | ..... | ..... |
| K | On | 24.7 | 16.6 | ..... | 20.7 | 16.0 | 16.8 | 16.5 | 10.4 | 14.9 |
| | Off | 60.0 | 44.4 | ..... | 52.2 | 88.4 | ..... | 76.3 | 123.7 | 96.1 |
| S | Not used | 39.0 | 33.2 | 28.4 | 33.5 | 40.6 | 52.1 | 32.9 | ..... | 41.9 |

* Larvae per gram/eggs per gram (exclusive of Strongyloides), × 100.

salt mixture was available throughout the one-year observation period. In spite of the intensive medication program from May through August, 1954, the average Haemonchus egg count reached a high of about 8,000 in September. At this time, an unexplained drop in the egg count occurred, but by November it had returned to an average of 5,500. The administration of a Cunic drench in November reduced the average count to 100 e.p.g. Of interest was the build-up of Strongyloides, which was also observed in the ram lambs on this farm.

The ram lambs (graph 7) were given 11 drenches (8 phenothiazine-lead arsenate, 1 copper sulfate, 2 Cunic) and the phenothiazine-salt mixture was available from May through November, 1954. The August 2 phenothiazine drench was quite effective in reducing the average egg count, whereas the June 30, August 25, September 28, and March 14 drenches were followed by increases in the egg counts. In the case of the June 30 drench, however, the first post-treatment reading was made 28 days after treatment. This would have been sufficient time for worms acquired after treatment to become sexually mature (patent). The copper sulfate and Cunic drenches were consistently followed by reductions in the egg count averages.

The results of the larval cultures are summarized (table 1). The average percentage of larval development of each animal and each flock is listed. The data from




Graph 4—Effect of phenothiazine-salt mixture and copper sulfate-nicotine sulfate drenches on the average nematode egg counts (eggs per gram of feces) of farm K lambs. Egg counts were made at approximately two-week intervals.

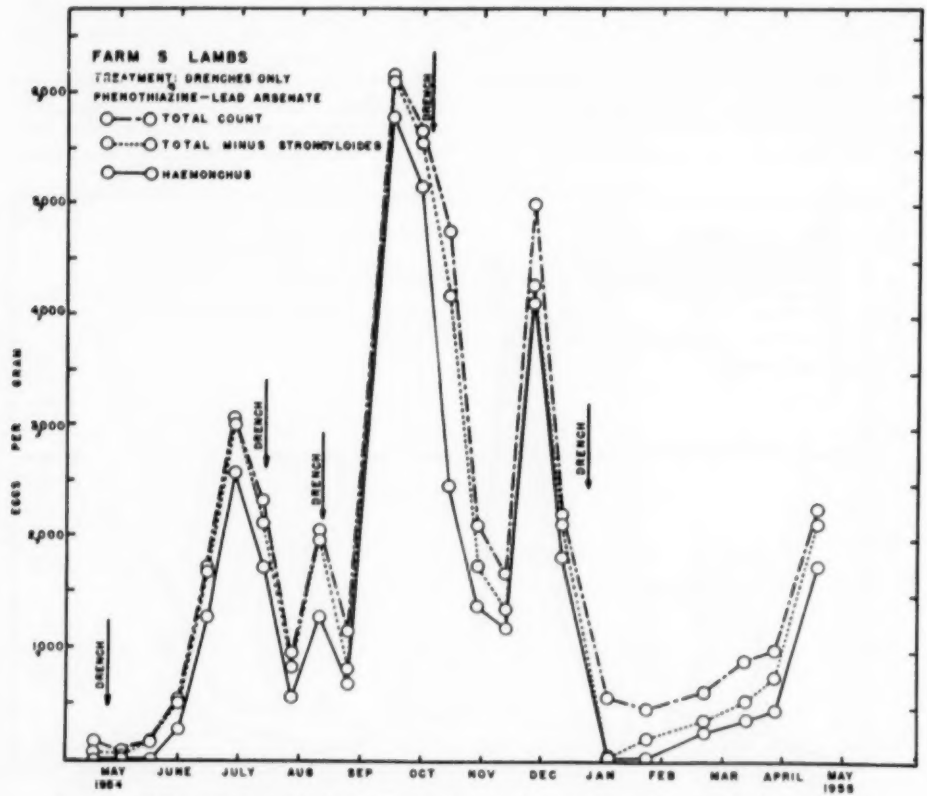
cultures made within one week following a drench were not included in the averages. The cultures were made from portions of the same fecal sample collected for the egg counts.

Cultures were made from 2 lambs from farm K during July through March, and the flock average while on the phenothiazine-salt mixture was 20.7 in contrast to 52.2 per cent larval development while off the mixture. Cultures from 3 lambs from August to December on farm S resulted in an average of 33.5 per cent development. On farm B, cultures from 3 lambs from July through March, while the phenothiazine-salt mixture was available, gave an average of 50.0 per cent development. Since the ewe lambs were on the salt mixture throughout the observation period, only the averages for the ram lambs for the period off the salt mixture were available. This was 65.8 per cent development as compared with the average of 74.0 per cent while on the mixture.

Cultures from 4 farm K breeding ewes were made from May through March. The average development was 14.9 per cent while on the salt mixture and 96.1 per cent while off the salt mixture. Cultures made from May through March from 3 breeding ewes from farm S resulted in an average of 41.9 per cent development. Cultures from 3 farm B ewes from May through August, the entire time of which the salt mixture was available, averaged 43.9 per cent development.

## DISCUSSION

Usually, it is not possible to arrange the experimental design of field studies in

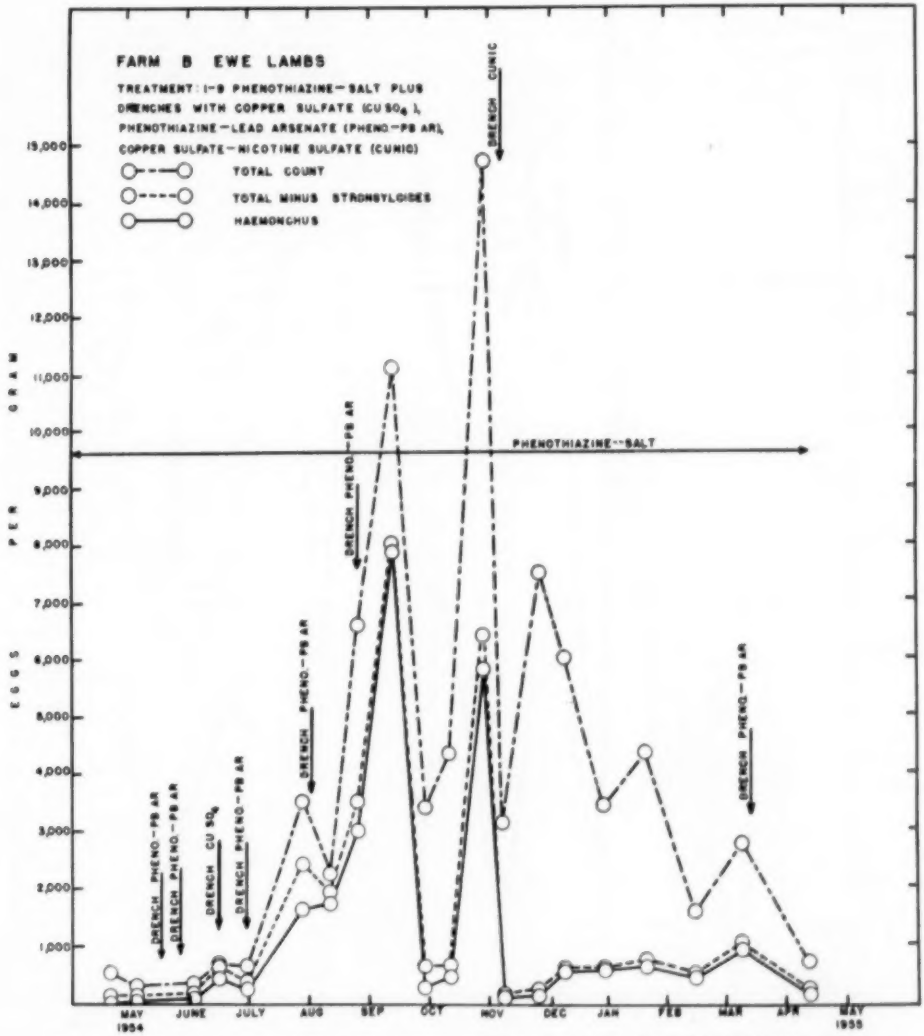



Graph 5—Effect of phenothiazine-lead arsenate drenches on the average nematode egg counts (eggs per gram of feces) of farm S lambs. Egg counts were made at approximately two-week intervals.

order to control the many variables that exist. Nevertheless, field studies are important, in that they correlate the usually more technical laboratory experiments with the practical procedures employed in the field.

In the present study, an attempt was made to evaluate, by periodic parasite egg counts and larval cultures, the medicinal control procedures of the three farms using various combinations of phenothiazine.

It is difficult to draw conclusions regarding the relative values of the phenothiazine-salt mixture on the various farms since the daily salt consumption of the individual animals was not measured and, therefore, the individual daily intake of phenothiazine was not known. However, on the various farms the salt mixture was consumed in approximately the recommended

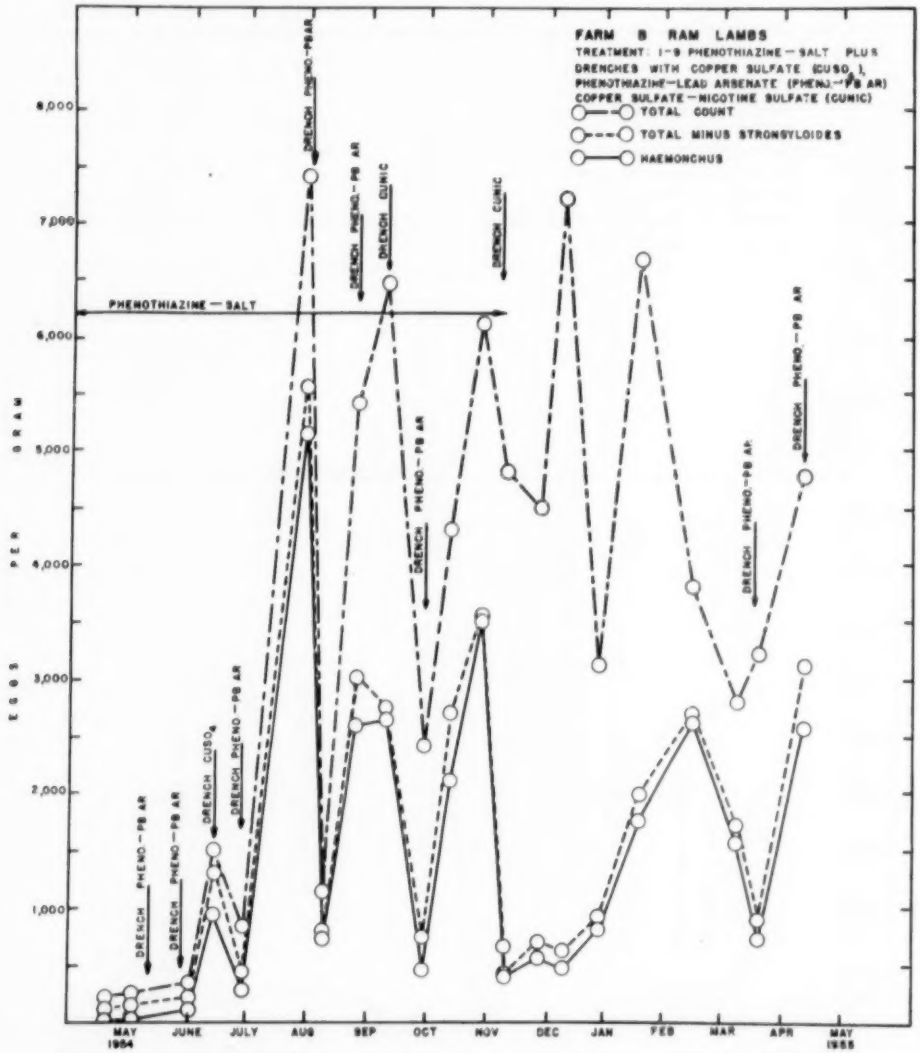



Graph 6—Effect of phenothiazine-salt mixture and copper sulfate, phenothiazine-lead arsenate, and copper sulfate-nicotine sulfate drenches on the average nematode egg counts (eggs per gram of feces) of farm B ewe lambs. Egg counts were made at approximately two-week intervals.

daily dose (0.5 Gm./head) as estimated by total flock consumption.

It is of practical significance that: (1) the egg count curves of farms S and B ewes were quite similar although the phenothiazine-salt mixture was not used on farm S; (2) the average egg count of farm K ewes reached its highest peak while the medicated salt was available (just how much higher the average count would have been in the absence of the salt mixture was not determined); and (3) despite the repeated drenchings of farm B lambs, high egg count averages developed while the salt mixture was available.

With respect to larval development, sheep on Farm K showed definite beneficial results from the use of the salt mixture as




Graph 7—Effect of phenothiazine-salt mixture and copper sulfate, phenothiazine-lead arsenate, and copper sulfate-nicotine sulfate drenches on the average nematode egg counts (eggs per gram of feces) of farm B ram lambs. Egg counts were made at approximately two-week intervals.

measured by the increased larval development when the drug was removed. Although data are insufficient for the period during which farm B sheep were off the medicated salt, a comparison of farm B with farm S averages reveals that a higher percentage of the larvae were developing on farm B when the drug was available than on farm S where phenothiazine-salt was not used. A comparison of farm B lambs while on the drug, with farm K lambs while off the drug, indicates the percentage development averages were about the same (50.0 and 52.2%, respectively). It should be emphasized again that the salt consumption of the individual lambs was not measured, therefore, the exact amount of phenothiazine consumed by each animal is not known. Further, to what extent the parasitism of farm B or K lambs would have developed in the complete absence of the salt mixture was not ascertained in the present field trials. Certain practical implications, however, can not be ignored, *i.e.*, the case of farm B where, with the phenothiazine-salt available and with an extensive phenothiazine drenching program, egg counts and larval development appeared to be in excess of what would be expected.

Haemonchus predominated on all three farms (graphs 1–7) throughout the observation period, except on farm B, where the lambs showed a build-up in Strongyloides. Previous reports [1, 9, 12] indicated that 0.1 Gm./lb.-therapeutic doses of phenothiazine would, in most cases, remove all *Haemonchus contortus* from adult sheep. This is considerably below the comparable dose level of 0.25 Gm./lb. used on farms S and B. Further, Gordon [5] found phenothiazine in doses of 0.6 Gm./kg. (0.27 Gm./lb.) highly efficient in destroying immature, 10- and 15-day-old *H. contortus*. Therefore, the egg count following a therapeutic dose of phenothiazine would not be expected to show a substanial increase before a time had elapsed equal to the prepatent period of worms less than 10 days old; and, since peak egg production occurs 25 to 35 days after infection, egg counts made as late as 14 days after drenching should still show the activity of the drug if the treatment was effective.

The present observations, without exception, associated a marked reduction in egg count averages following therapeutic treatment with Cunic of farm K ewes and lambs, and farm B lambs. Slight egg count reductions were observed following the use of copper sulfate in farm B ewe and ram lambs. In the case of farm S, where phenothiazine drenching was the sole medicinal control, the egg count averages were reduced following treatment almost without exception. On the other hand, when phenothiazine drenches were used on the farm B ewes, ewe lambs, and ram lambs, not only did the average egg count fail in nine cases to show decreases, but in many showed marked increases.

Considering the extensive drenching program on farm B, the apparent failure of the phenothiazine drenches (in contrast to the success with Cunic and the favorable results obtained with phenothiazine drenching on farm S) would seem to indicate a drug tolerance of the worms. This would be in keeping with the controlled laboratory experiments reported in a previous paper [8] in which this same strain of worms was found to have a threshold four to eight times higher than a control strain. Further, phenothiazine, both in salt mixtures and in drenches, has been the essential parasite control medication utilized on farm B for the ten years preceding the study.

## Summary and Conclusions

Three farms in the central Kentucky area, which used different medicinal procedures with varying degrees of phenothiazine for the control of nematodes in sheep were selected and the parasite picture, as indicated by worm egg counts and larval cultures, was followed for an 18-month period.

On farm K, drenching with copper sulfate-nicotine sulfate was followed by a reduction in the worm egg counts in every instance. While a high average egg count developed when phenothiazine-salt was available, the drug was effective in reducing larval development.

With phenothiazine drenching as the sole medicinal control, effective reduction in the egg count averages was observed on farm S.

On farm B, where the most extensive drenching program was employed, only the copper sulfate-nicotine sulfate drench was consistently effective. When phenothiazine drenches were used, the average egg count failed to decrease in nine instances, and, in several cases, substantial increases followed treatment. The percentage of larvae developing in farm B lambs when phenothia-

zine-salt was available was about the same as in farm K lambs (while off the drug) and higher than in lambs on farm S, where this mixture was not used.

Considering (1) the extensive drenching program utilized on farm B; (2) the apparent failure of the phenothiazine drenches on farm B, while the same dose rate and commercial preparation of phenothiazine was successful on farm S; (3) the consistent success with Cunic drenches on farm B; (4) the comparatively high percentage of larval development on farm B with phenothiazine-salt available; and (5) a ten-year history of extensive phenothiazine utilization by farm B; it would seem that the worms from farm B had developed a tolerance to the drug. This observation is compatible with the controlled laboratory findings for the same strain of worms (strain B) reported in the earlier papers of this series. These observations are not regarded as sufficient evidence to justify general discontinuance of phenothiazine drenches or salt mixtures. However, it would be advisable, especially in flocks with a history of parasite problems, to alternate phenothiazine with Cunic drenches and to make the salt mixture available.

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# Symmetrical Cortical Siderosis of the Kidneys in Goats

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THIS PAPER reports further investigations into the occurrence of symmetrical cortical siderosis of the kidneys in the goats of northern Iraq.[46] There appears to be no similar condition reported in medical or veterinary medical literature. The object of our investigation was to discover why this condition is confined to goats and why it appears to be found only in northern Iraq. The mountains of northern Iraq and the borders of Persia, which are accessible only to goats, are rich in copper and iron compounds but apparently lack cobalt, without which iron can not be assimilated by the body.[16] The vitamin $B_{12}$ requirement is higher for sheep and goats than for other animals, but normally, when there is adequate cobalt in the diet, it is met by bacterial synthesis in the rumen.[29]

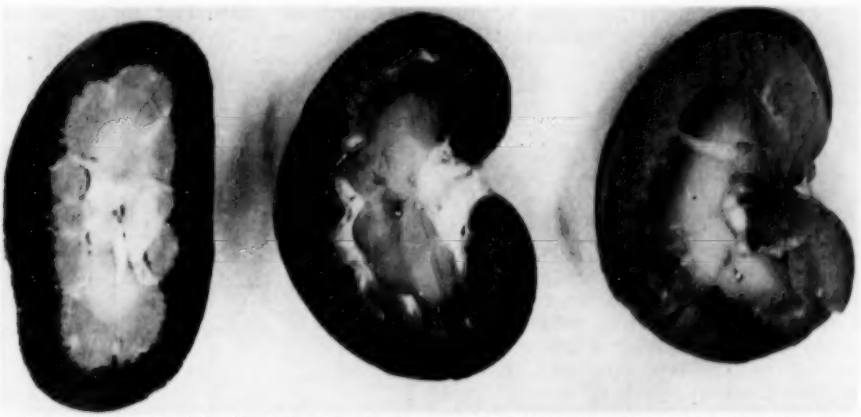

**Fig. 1 (Left to right)—Typical goat kidney showing cortical siderosis; cortical siderosis after massive staining with Prussian blue; normal goat kidney for comparison.**

## INCIDENCE

This disease was present in 26 (3.6%) of 729 goats examined in the slaughterhouse. The ages of the diseased goats were from 2 to 5 years, and more females than males were affected.

The kidneys, liver, spleen, pancreas, testes, lymph nodes, and suprarenals of 12 goats were examined.

At necropsy, the pigmented kidneys were found to be slightly enlarged and without capsular adhesions; the surfaces were smooth. There were no hemorrhages or areas of infarction. The characteristic microscopic finding was a deposition of hemosiderin in the kidney cortex, causing it to be a deep brown or blackish color (fig. 1). In mild cases of this condition the iron was located inside epithelial cells. In heavily pigmented kidneys, the epithelial cells of the proximal convoluted tubules showed both diffuse and granular forms of iron deposition, accompanied by cloudy swelling of the epithelial cells with some nuclear degeneration. The lumens of the tubules were filled with desquamated cells and homogenous fluid, giving a faintly positive reaction with Prussian blue. Even in these heavily pigmented kidneys, there were no lesions in the glomeruli or the collecting tubules.

The liver showed cloudy swelling with iron pigment inside Kupffer cells and, rarely, in hepatic cells (fig. 4). There was dilitation of the central veins, but no evidence of any inflammatory process.

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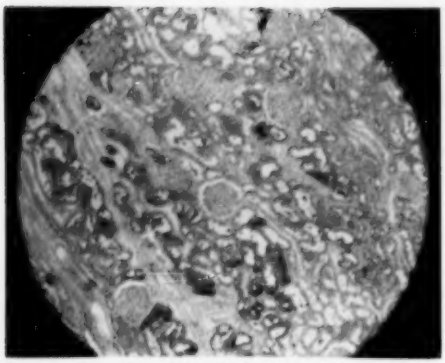

**Fig. 2—Iron deposition in the convoluted tubules, glomeruli, and medullary tubules, free of pigments. Stained with Prussian blue and hematoxylin-eosin; x 70.**

Iron pigment was also found inside the reticuloendothelial cells of the sinusoids and pulp of the spleen (fig. 5), and in the sinusoids and reticuloendothelial cells of lymph nodes.

An unexpected finding in male animals was complete or partial necrosis of the epithelial cells of the seminiferous tubules of the testes (fig. 6), the interstitial cells being normal. The testes gave a positive Prussian blue test. Testicular atrophy occurred in 7 of 12 goats.

The suprarenals, pancreas, and lungs showed negligible changes or were normal in structure.

## ETIOLOGY

From preliminary chemical analyses, we suspected that the cause of these lesions of the kidney and testes was excessive iron and copper deposition in the tissues. Chemical examination appeared to confirm this, for high levels of copper were found in all the viscera with particularly large amounts in the testes. Experiments on feeding copper and iron to goats were carried out to test this hypothesis but they did not produce lesions in the cortex similar to those of spontaneous bilateral cortical siderosis. There may, therefore, be additional dietary factors in the cause of the disease. Possibly a deficiency of cobalt is such a factor. Soil samples, brought from northern regions of Iraq, contain copper compounds up to a level of 4 per cent and inorganic iron compounds up to a level of 25 to 30 per cent. The goats in search of food in these regions dig into soil rich in inorganic iron and copper compounds in order to get the roots of the plants, which contain them, especially in winter. Other foodstuffs eaten by goats include spinach, millet, parsley, and mustard, a diet which is rich in iron, but poor in protein.[33] Moreover, these goats live on dry food collected from ground deficient in cobalt. The iron compounds found in these foods probably pass through the intestinal wall unchanged, to reach the kidneys. In the kidneys, the iron passes through the glomeruli to be deposited inside the cells of the proximal convoluted tubules in the same way that injected carbon particles do.[34, 43] It may be that in this way the tissue changes are more or less limited to the cells of the proximal convoluted tubules.

The absence of necrosis and hemorrhage indicates that neither spasm, toxemia, nor any vascular thrombosis is present. The presence of ferric compounds in the cells of the proximal convoluted tubules is irreversible and may be the cause of the hypertrophy of the basement membrane. The iron does not apparently cause any disease or functional discomfort to the animals, unless it passes as ferritin to the blood, when it becomes harmful.[31, 32] The ferritin is supposed to be found in connection with hemosiderin.[18, 21]

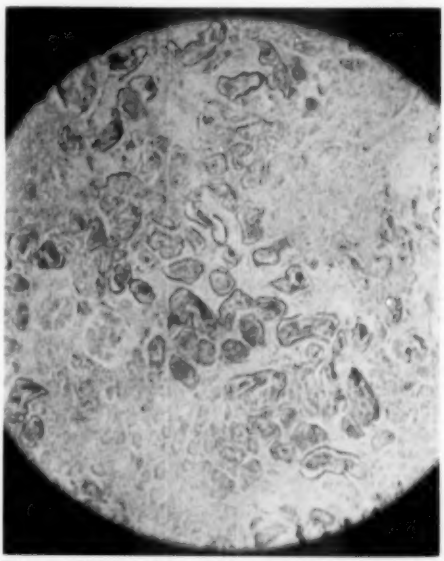

**Fig. 3—Thickened basement membrane with abundant iron in convoluted tubules. Stained with Prussian blue and safranin; x 70.**

Continued injection of blood into rabbits (as well as prolonged periods of oral administration of iron, and injection of colloidal iron, to mice) produces hemosiderosis but not hemochromatosis.[28]

Finch *et al.*[12] demonstrated the storage of iron in rats fed a diet deficient in phosphates.

Kinney *et al.*[25] produced hemosiderosis in rats after administration of a diet deficient in protein, minerals, and vitamins but containing large amounts of ferric citrate.

Sheldon[30] observed that hemosiderosis rather than hemochromatosis was found in certain iron-storage states such as hemolytic anemia, malaria, and congenital hematoporphyrinuria. He also observed hemochromatosis as a congenital inadequacy in the metabolism of iron.

## Materials and Methods

Eight goats, two groups of 4 each, were explored surgically, by lumbar incision, and found to have normal unpigmented kidneys.

The first group was given 10 Gm. of a soil dust containing inorganic iron (30%) and copper (4%) daily, with the ordinary food, for four months. The second group was given 15 Gm. of the same soil dust daily, with a corn grit diet which was deficient in protein, for three months.

## Results

The laboratory findings in these animals, and in a group of 6 normal goats, are summarized (table 1).

The goats fed on their ordinary diet with extra iron and copper salts did not develop cortical coloration or tissue siderosis; while goats fed on corn grit, together with iron and copper salts, developed siderosis of the convoluted tubules, liver, and spleen. This, however, was not similar to the spontaneous deposition of iron in the kidney cortex, being much less extensive and not giving rise to a black coloring of the kidney which is apparent to the naked eye. There may thus be a further factor in the disease, responsible for the discoloration, perhaps absence of cobalt. This condition differs from that of liver cirrhosis for, in cases of spontaneous cortical siderosis, no trace of liver cirrhosis could be found and, moreover, it did not resemble hemochromatosis, for neither diabetes mellitus nor skin pigmentation was present.

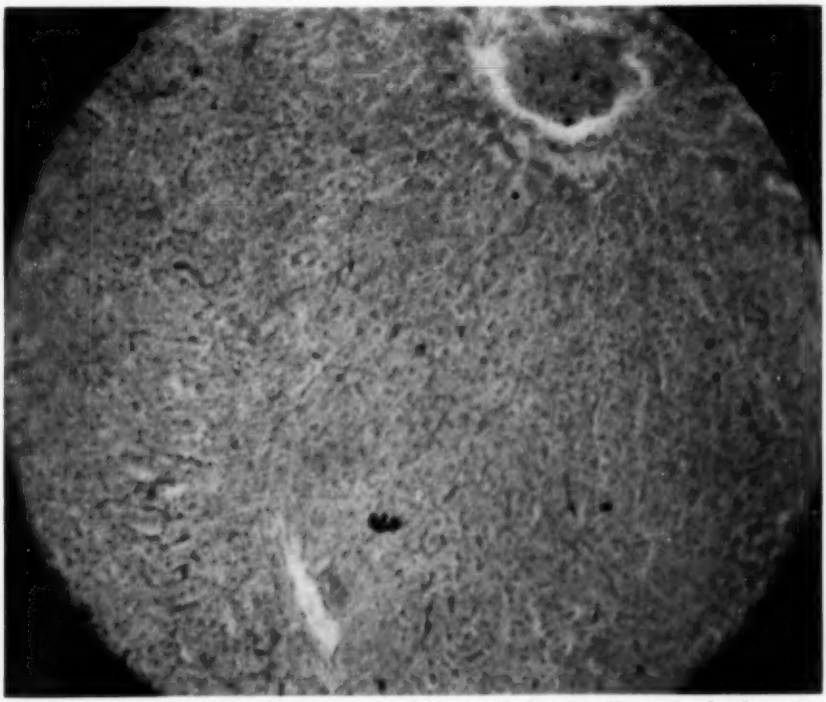

Fig. 4—Iron pigment inside Kupffer's cells. Stained with Prussian blue and safranin; x 50.

TABLE 1—Blood Chemical Values of Apparently Normal Goats and of the Two Experimental Groups Given Additional Iron and Copper*

| Tests | Normal animals | | | | | | | First experimental group given small doses of iron and copper | | | | | Second experimental group of goats after treatment with large doses of iron and copper | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | Av. | 1 | 2 | 3 | 4 | Av. | 1 | 2 | 3 | 4 | Av. |
| Total protein (Gm./100 ml.) | 6.4 | 6.5 | 5.5 | 6.5 | 6.5 | 5.7 | 6.2 | 5.2 | 5.2 | 5.5 | 5.5 | 5.4 | 3.8 | 5.5 | 5.0 | 5.5 | 4.9 |
| Albumin (Gm./100 ml.) | 4.0 | 4.0 | 3.6 | 3.0 | 3.5 | 3.0 | 3.5 | 2.4 | 2.0 | 2.6 | 2.0 | 2.3 | 1.8 | 3.6 | 3.5 | 3.8 | 3.2 |
| Globulin (Gm./100 ml.) | 2.5 | 2.5 | 1.9 | 3.5 | 3.0 | 2.7 | 2.7 | 2.6 | 3.2 | 2.9 | 3.5 | 3.1 | 2.0 | 1.4 | 1.8 | 1.7 | 1.7 |
| Cephalin cholesterol turbidity test | 3+ | 3+ | 3+ | 3+ | 3+ | 3+ | 3+ | 3+ | 3+ | 3+ | 3+ | 3+ | 5+ | 3+ | 4+ | 4+ | 4+ |
| Thymol turbidity (units) | 2.5 | 2.0 | 3.0 | 3.5 | 4.0 | 2.0 | 2.8 | 2.0 | 3.0 | 1.0 | 1.0 | 1.8 | 5.0 | 3.0 | 4.0 | 3.0 | 3.8 |
| Miller test | — | — | — | — | — | — | .... | — | — | — | — | .... | + | — | — | + | .... |
| Van den Bergh | — | — | — | — | — | — | .... | — | — | — | — | .... | Biphasic | — | — | — | .... |
| Blood urea N (mg./100 ml.) | 32 | .... | .... | 26 | .... | 34 | .... | 28 | .... | .... | 32 | .... | 300 | 50 | 70 | 50 | .... |
| Icteric index (units) | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | 35 | 9 | 9 | 7 | .... |
| Bilirubin (mg./100 ml.) | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | 13.0 | 0.7 | 0.8 | 0.7 | .... |
| Histological examination | The visceral lesions were very slight. Congestion and, rarely, cloudy swelling were seen in the kidneys. Hemosiderin was absent or faintly positive for Prussian blue reaction. Characteristic bilateral cortical siderosis was not seen in any kidney. | | | | | | | .... | .... | .... | .... | .... | Hemosiderin was noticed in the kidneys, particularly in the convoluted tubules, and also in the reticuloendothelial and cord cells of the liver and spleen pulp. The testicles were atrophied and the seminiferous tubules degenerated. Hemosiderin was marked in connective tissue, less in cells. There was marked congestion with swelling or necrosis of parenchymatous organs. | | | | |

* Analyses made by Dr. J. Shabi. † Direct.

Although deposition of iron in the proximal convoluted tubules occurred in the second group, none showed the gross appearance of bilateral cortical siderosis similar to spontaneous lesions. Lesions suggestive of renal periarteritis nodosa were, however, observed.

A group of goats found to have pigmented kidneys were examined. Various tests were carried out, including some made on blood, urine, and feces, and the results compared with those of tests made on a group of goats with unpigmented kidneys. The animals with discolored kidneys appeared to be healthy and the laboratory findings showed that the pigment had caused no ill effects. The blood urea was not raised and the urine was free from glucose and iron pigment. The presence of albumin in the urine was due to a urinary tract infection and was found in goats with normal, as well as those with colored, kidneys. Thymol turbidity, cephalin flocculation, and the albumin-globulin ratio of the blood protein were normal and did not differ much from those found in the normal goats. The number of blood leukocytes and erythrocytes, the hemoglobin levels, and the blood urea were within normal limits (tables 2–5).

Chemical analyses were made of the tissues of normal and pigmented cortices, and a number of other tissues (tables 5 and 6).

The radiographs of normal and pigmented kidneys were similar.

## DISCUSSION

Very little has been published regarding hemosiderosis in animals. Iron deposits may be seen in the reticuloendothelial systems of old horses. In fowl, iron deposits may be found in glial cells.[24] Pathological conditions found in animals and man in which hemosiderosis occurs include:

1) Local and general blood circulatory troubles occur as in the case of hemorrhage or stasis (these were excluded from the present syndrome).

2) Piroplasmosis—there was no evidence of this in blood smears made from these goats. In this condition, iron is deposited particularly in the liver, spleen, bone marrow, and lymph nodes. In the kidney there is involvement of the cells of Henle's loop but not of the cortex.

3) In the group of diseases associated with muscle degeneration, there is free myoglobin and myosiderosis, especially in the kidneys. The clinical and pathological findings associated with

**TABLE 2—Results of Blood Examination and Laboratory Tests of 30 Clinically Normal Goats and 2 (Goats 1 and 2) with Pigmented Kidneys**

| Tests | Normal goats | Goat 1 | Goat 2 |
|---|---|---|---|
| Erythrocytes | 10,000,000–13,000,000/cmm. | 15,000,000/cmm. | 14,000,000/cmm. |
| Leukocytes | 10,000–14,000/cmm. | 14,400/cmm. | 14,000/cmm. |
| Hemoglobin (Sahli) | 60–65 mg./100 ml. | 67 mg./100 ml. | 62 mg./100 ml. |
| Van den Bergh | All negative | Neg. (direct) | Neg. (direct, indirect) |
| Mean corpuscular vol. | 32–36 mg./100 ml. | ..... | ..... |
| Bilirubin level (mg./100 ml.) | ..... | 0.5 | 0.5 |
| Spectroscopic exam. | ..... | Normal Hb. (D-E bands) | Normal Hb. (D-E bands) |
| Icteric index (units) | ..... | 2 | 2 |
| Blood urea N (mg./100 ml.) | ..... | 36 | 32 |
| Packed erythrocyte volume (mg./100 ml.) | ..... | 41 | 44 |
| Fragility test (% NaCl) | ..... | Complete—0.60–0.65<br>Partial—0.70 | 0.55–0.60<br>0.65 |
| Donath-Landsteiner | ..... | — | — |
| Rosenbach | ..... | — | — |

them are not, however, in accordance with our observations.[13]

4) In human paroxysmal hemoglobinuria, there are: (1) stigmata of syphilis, often congenital; (2) the attacks can be induced by immersing the hands or feet in cold water for ten to 20 minutes; and (3) the Donath-Landsteiner reaction is positive. The Donath-Landsteiner reaction was found to be negative in 2 goats with siderosis.

**TABLE 3—Urinalysis of 10 Clinically Normal Goats and of 2 (Goats 1 and 2) with Siderosis**

| | Normal goats | Goat 1 | Goat 2 |
|---|---|---|---|
| Reaction | Alkaline 6–Acid 4 | Acid | Alkaline |
| Albumin | Trace 6–Neg. 4 | Trace | Trace |
| Sugar | Nil 10 | Nil | Nil |
| Urobilin | ..... | Nil | Nil |
| Bile | ..... | Nil | Nil |
| Hemosiderin | ..... | Nil | Nil |
| Bilharzia | Neg. 10 | ..... | ..... |
| Urinary sediment | | | |
| Pus cells | Moderate 6–Neg. 4 | Few | Few |
| Renal casts | Positive 2–Neg. 8 | Nil | Nil |
| Erythrocytes | ..... | Few | Few |

5) Hemochromatosis occurs.[14, 28]

6) In bilateral cortical siderosis, the iron pigment is deposited in the proximal convoluted tubules. In the goats tested in the present experiment, there were no marked changes in the liver, pancreas, or spleen. Skin pigmentation was absent and the Prussian blue test of the skin was negative. No signs of diabetes mellitus were observed. Therefore, this condition in goats resembles neither hemochromatosis nor Bantu siderosis.

7) Mineral poisoning with bismuth, lead, antimony, mercury, or silver was excluded as a possible condition of the goats in the present experiment, by chemical examination.

8) Parasitic diseases, bilharziasis and *Bacillus welchii* infection,[4] were eliminated as factors in the present situation by direct and cultural methods.

**TABLE 4—Examination of the Stools of 6 Clinically Normal Goats and of 2 (Goats 1 and 2) with Pigmented Kidneys**

| | Normal goats | Goat 1 | Goat 2 |
|---|---|---|---|
| Occult blood | | | |
| (benzidine test *) | 5+ 1— | + | + |
| (guiacum test) | ..... | + | + |
| Pus cells | 3+ 3— | + | — |
| Mucus | 2+ 4— | — | + |
| Erythrocytes | 2+ 4— | + | — |

* Gum guiacum gave less positivity than the benzidine test.

Other diseases, similar to those found in man, are concerned with abnormal states of serum iron.[6, 8, 27] In general, a high serum iron level occurs in those diseases in which either the total iron is high in the body, e.g. in hemochromatosis or transfusion hemosiderosis, or the erythrocytes are fragile and easily hemolyzed, e.g. in hemolysis and the Mediterranean anemias. A high serum iron concentration also occurs in a third group of diseases in which the marrow appears to demand iron but can not use it readily, e.g. in pernicious anemia or pyrodocin deficiency. There are also those conditions in which the liver

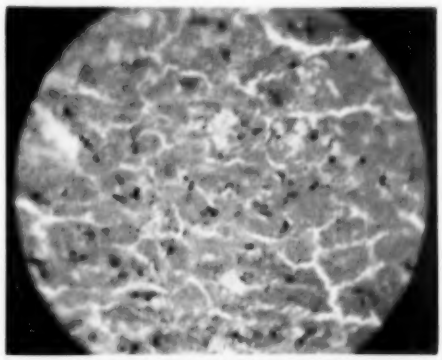

**Fig. 5—Iron pigment inside reticuloendothelial cells of spleen. Stained with Prussian blue and safranin; x 75.**

**TABLE 5—Chemical Analysis of Normal and Pigmented Cortex of Goat Kidneys***

| Element | Normal cortex | Pigmented cortex |
|---|---|---|
| Phosphorus | 0.026 | 0.268 |
| Iron | 0.038 | 0.194 |
| Copper | 0.011 | 0.040 |

* This analysis was made by Dr. R. Dajani.

is damaged and can not store excess iron, e.g. in cirrhosis or hepatitis.

It can be seen that the features of the kidneys which we describe in these experiments are not in accordance with any of the above diseases; they more closely resemble hemochromatosis and the hemosiderin nephrosis of paroxysmal hemoglobinuria.[22, 44] The etiological factor is probably nutritional. Copper is found in increased amounts in nearly all viscera and may also be the cause of the atrophy of the testes found in these goats. Our findings indicate an increase in the amount of copper in various organs and also in the blood, as compared with the controls.

Paulais[36] found that the amount of copper was higher in pigmented testes than in unpigmented ones. This may explain the high incidence of atrophy of the testicles in goats with pigmented kidneys. The adrenal glands seemed to be normal in cases of pigmented kidneys.

It is possible that in this condition we are dealing also with a state of cobalt deficiency. Animals exposed to severe cobalt deficiency become progressively weakened, show muscle atrophy, their coats become rough, and they develop marked anemia with hypoplastic marrow. Hemosiderosis

**TABLE 6—Analysis of Tissues from Normal and Pigmented Goat Kidneys***

| | Iron | | Copper | |
|---|---|---|---|---|
| Substance | Normal (mg./ 100 cc.) | Pigmented (mg./ 100 cc.) | Normal (mg./ 100 cc.) | Pigmented (mg./ 100 cc.) |
| Blood | 0.0700 | 0.0725 | 0.0012 | 0.0017 |
| | 0.0680 | 0.0728 | ..... | ..... |
| Kidney | 0.0300 | 0.0712 | 0.0021 | 0.0026 |
| | 0.0340 | 0.0619 | 0.0020 | 0.0022 |
| | 0.0292 | 0.0780 | 0.0026 | 0.0029 |
| | 0.0410 | 0.0680 | 0.0012 | 0.0020 |
| Spleen | 0.0418 | 0.0703 | 0.0012 | 0.0020 |
| | 0.0502 | 0.0676 | 0.0012 | 0.0016 |
| Liver | 0.0330 | 0.0640 | 0.0022 | 0.0026 |
| | 0.0400 | 0.0791 | 0.0026 | 0.0031 |
| Testicles | 0.0272 | 0.0287 | 0.0020 | 0.0040 |
| | 0.0346 | 0.0369 | 0.0018 | 0.0036 |
| Serum | 0.0460 | 0.0600 | ..... | ..... |

* Analyses were made by Dr. Hassan Al Rabati using the methods of Loiseleur, and Lachiver and Leloup.[28]

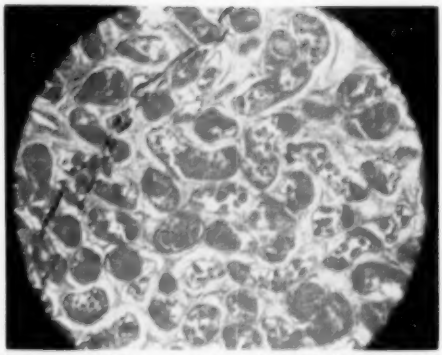

**Fig. 6—Diffuse necrosis of testicular tubules. Stained with Prussian blue and safranin; x 75.**

develops because of the inability of the marrow to use iron for hemoglobin synthesis.[6, 9, 19, 30] The livers of sheep deficient in cobalt contain 0.02 p.p.m. dry weight of cobalt as contrasted with 0.12 p.p.m. in control animals. Horses, swine, rabbits, guinea pigs, and rats all appear to be unaffected by cobalt deficiency.[3] Apparently the ruminating animals, especially sheep and goats,[40] have a high requirement for vitamin $B_{12}$. These facts may explain our observation that goats are affected by this condition while other animals are not.

## SUMMARY AND CONCLUSIONS

The unique features of spontaneous bilateral siderosis in the renal cortex of goats from northern Iraq are described. Iron deposition is rare or absent in organs other than the kidney. Copper is found to be increased in various organs, particularly in the testes, which suggests that increased amounts of this element cause the observed high incidence of testicular atrophy.

This condition seems to occur only in the mountainous area of northern Iraq, which is rich in copper and iron but deficient in cobalt and is reached only by goats. The deposition of ferric compounds in the proximal convoluted tubules does not, of itself, cause clinical manifestations, dysfunction, or important histological changes, except thickening of the basement membrane.

The findings in the urine, stools, blood, and serum of both goats with normal and with pigmented kidneys are recorded.

Goats, fed low doses of inorganic iron

and copper together with ordinary feed, revealed no functional or anatomical disorder; those fed high doses, with protein deficient diets, showed generalized siderosis of the viscera, particularly the kidney, with parenchymatous changes in various organs. Neither group developed lesions similar to those of spontaneous symmetrical cortical siderosis. Some other factor, perhaps a deficiency of cobalt, may be required to produce this condition.

Iron deposition in the convoluted tubules produces no functional renal disturbances; therefore, the affected animals show no diagnostic clinical or laboratory findings. The role of copper in the production of the disease is not known.

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# Studies on Genital Vibriosis in Sheep

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FROM THE literature, there appear to be differences betwen bovine and ovine vibriosis with respect to the route of infection, clinical signs, and course of infection in a herd. In cattle, the transmission is first and foremost coital, while in sheep, this mode of infection appears to be less important.[6, 7, 12, 19, 21]

Whether the infection may be transmitted from cow to ewe, and vice versa, seems to be somewhat obscure.[5, 9] It seems to be clear that the infection has little chance to be carried over to the following season.[1, 2, 16, 18]

In cattle, the most pronounced sign is a marked necessity for repeated breeding. In the ewe, this has not been described, the most characteristic and common sign being abortion in the late stages of pregnancy.

This is a report of an investigation of an experimental sheep herd, where a spontaneous infection with vibriosis occurred.

## MATERIALS

In the spring of 1956, a number of abortions due to vibriosis occurred in the ewes at the State Veterinary Experimental Farm for Sheep (Norway). Experienced personnel attend these ewes and the farm has constant contact with veterinarians. The animals are out of doors except during midwinter, when they are indoors and are given supplementary feed. Usually, about 180 animals are retained over the winter. All the animals were numbered, and all services carefully controlled and recorded. The rams were not turned out with the ewes during the service season.

During the spring, 18 of 142 ewes aborted between the ninety-second and 140th days of pregnancy; the majority with 2 or 3 fetuses.

Most of the sheep were kept, in groups, in large box stalls with earth floors. The first ewes that aborted remained in their groups for about two weeks. Later, however, all aborting ewes were isolated.

Vaginal mucus was taken from aborting ewes some time after delivery, and from pregnant healthy ewes and young unmated female sheep (gimmers). In addition, samples were taken from fetal fluid at normal parturitions, from the gastric contents of newborn normal lambs, and from semen of the rams used. Five lambs, stillborn or dying shortly after full-term pregnancy, were also investigated.

## METHODS

*Collection and Inoculation of the Material.*—Inoculation on thiol medium was made from the stomach, liver, and other organs, and also from the heart blood of all the aborted fetuses. The thiol tubes were incubated at 37 C. in 10 per cent $CO_2$ atmosphere and examined after four, seven, and ten days in the phase-contrast microscope. From the majority of fetuses, inoculations were also made on blood agar incubated at 37 C. and on half-fluid medium incubated at 22 C. Phase-contrast microscopy of the gastric contents or the alimentary canal was always carried out.

Mucus from the vagina, usually small quantities, was collected with a sterile glass pipette and immediately inoculated on thiol medium.

Fetal fluid was obtained by puncturing the fetal sac with a sterile needle as soon as it appeared in the vulva. The gastric content of the lambs was collected, immediately after birth, with a syringe and a small stomach tube.

The material obtained was inoculated in thiol tubes. As a rule, phase-microscopy was also used.

Semen from the rams was collected with a sterile artificial vagina.

*Isolation of Vibrio.*—The method of the Pasteur pipette, as proposed by Willems and described by Florent, was used (Lindqvist [14]). As a supplement to this method, cultures were occasionally made on blood agar, from which individual colonies were picked off.

*Biochemical Testing of the Vibrio Isolated.*—The pure cultures of bacteria were tested biochemically with reference to catalase activity, $H_2S$ production, nitrate reduction, and stab culture as described by Bryner and Frank.[4]

*Inoculation of Pregnant Guinea Pigs with the Vibrio Isolated.*—Guinea pigs, 5 to 6 weeks pregnant, were inoculated intraperitoneally with 1 cc. of a 72-hour Vibrio culture according to the method used at this laboratory since 1950.[8] Each strain tested was inoculated into 2 guinea pigs, with one exception in which only 1 guinea pig was used. As controls, 4 guinea pigs were inoculated with 1 cc. of sterile thiol medium.

Animals from which vibrios could be isolated from the inner organs of the guinea pig fetuses, and where the biochemical tests were positive, were considered infected.

*Serological Examinations of the Vibrio Strains Isolated.*—Antigens (O + H) were prepared from strains isolated from aborted fetuses, pregnant sheep, gimmers, ewes at normal parturition, and from semen.

The antigens were used to prepare O + H

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antiserums in rabbits that previously had not been found to produce normal agglutinins. The rabbits were immunized by injecting intravenously, on three consecutive days, 0.5, 1.0, and 1.5 cc., respectively, of the formol-treated antigen. After an interval of three days, they were inoculated with 0.5, 1.0, 1.5, and 2.0 cc. of the antigen, respectively, on four consecutive days. Immune serums were collected after another interval of seven days, and preserved with merthiolate (1:10,000).

Complete titration for each antiserum was performed with the homologous antigen, and cross-agglutination tests were made between each individual antiserum and all antigens. These antigens were also used for agglutination tests on *Vibrio fetus*-positive bovine vaginal mucus.

## RESULTS

*Aborted Fetuses.*—Vibrios were isolated, usually in large numbers and often in pure cultures, from all except 1 of the 18 ewes which aborted. They were easily detected by direct microscopy of the contents of the stomach and alimentary canal.

All fetuses aborted before the 110th day of pregnancy were stillborn. In the four abortions which occurred after the 127th day of pregnancy, the lambs were alive at birth, but soon died.

There were no demonstrable changes that clearly differentiated these aborted lambs from lambs aborted at the same stage of pregnancy from other causes.

*Vaginal Mucus.*—Vibrios could not be isolated from vaginal mucus obtained two days to four weeks after abortion in the first 13 ewes which aborted. They were demonstrated in material from the fetal membranes which had been retained for two days by 1 ewe.

Vaginal mucus was taken twice, first from 5 and, three weeks later, from all 25 gimmers. Vibrios were obtained from 7 but, in pure culture, from only 5.

Vibrios were isolated from 5 of 25 pregnant ewes selected at random. All lambed normally later.

*Normal Births.*—Fetal fluid from ewes, or gastric contents from newborn lambs, usually both, was taken at 72 normal births. Vibrios were isolated in pure culture in 3 cases, twice from fetal fluid, and once from stomach contents. All 3 lambs involved survived.

*Semen.*—Semen was collected several times during the spring and summer. Vibrios were isolated from 6 of the 11 rams. These 6 rams had all been cross-mated during the service season, the other 5 had not.

*Stillborn, Full-Term Lambs.*—Vibrios were not demonstrated in any of the 5 stillborn lambs investigated.

*Biochemical Examination of the Vibrio Strains Isolated.*—The results of the biochemical examination of 26 strains are shown (table 1). Nine strains isolated from aborted fetuses and one strain from a normal parturition were not tested since these cultures had died. It would appear that vibrios isolated from ewes are more difficult to keep alive in culture than bovine strains. During the latter part of these investigations, freeze-drying was used and it became easier to retain the strains.

**TABLE 1—Biochemical Tests of Vibrios Isolated from Sheep**

| Material | Strain | Catalase | $H_2S$ | $NO_2$ | Deep stab |
|---|---|---|---|---|---|
| Aborted fetuses | 100–51 | 3–4 mm. | – | + | – |
| | 180–49 | 1–2 mm. | – | + | – |
| | 105–50 | 2–3 mm. | – | + | – |
| | 185–54 | 1–2 mm. | – | + | – |
| | 146–51 | 3–4 mm. | – | + | – |
| | 145–51 | 3–4 mm. | – | + | – |
| | 149–53 | 2–3 mm. | – | + | – |
| Retained fetal membranes | 185–54 | 1–2 mm. | – | + | – |
| Vaginal mucus from pregnant ewes | 32–53 | 4 mm. | – | + | – |
| | 41–53 | 4 mm. | – | + | – |
| | 108–54 | 1–2 mm. | – | + | – |
| | 110–54 | 2 mm. | – | + | – |
| | 75–54 | 3 mm. | – | + | – |
| Vaginal mucus from virgin gimmers | 74–55 | 2 mm. | – | + | – |
| | 111–55 | 2–3 mm. | – | + | – |
| | 71–55 | 1–2 mm. | – | + | – |
| | 16–55 | 1 mm. | – | + | – |
| | 102–55 | 2–3 mm. | – | + | – |
| Normal parturitions | 22–53 | 2–3 mm. | – | + | – |
| | 229–50 | 1–2 mm. | – | + | – |
| Semen | M | 0 mm. | – | + | – |
| | 2–55 | 0 mm. | – | + | – |
| | P | 3 mm. | – | + | – |
| | H | 0 mm. | – | + | – |
| | 31–55 | 3 mm. | – | + | – |
| | 39–55 | 2 mm. | – | + | – |

The catalase activity was less than 5 mm. for all the strains. No strain showed $H_2S$ production, but the nitrate reduction was positive. In stab cultures, it was possible to observe growth only at the top of the medium, and this was considered negative.

All the isolated Vibrio strains reacted similarly to the biochemical tests.

*Inoculation of the Isolated Vibrio Strains in Pregnant Guinea Pigs.*—The results of these inoculations are shown (table 2).

No vibrios could be detected in the aborted fetuses (strains 146–51 and 229–50) in two cases of abortion in the inoculated guinea pigs. In one case (185–54), the fetuses were eaten before they could be

TABLE 2—The Abortifacient Effect on Pregnant Guinea Pigs of Vibrio Strains Isolated from Sheep

| Material | Strain | No. of guinea pigs injected | No. of abortions | No. of confirmed Vibrio abortions |
|---|---|---|---|---|
| Aborted fetuses | 180–49 | 2 | 2 | 2 |
| | 185–54 | 2 | 2 | 1 |
| | 146–51 | 2 | 1 | 0 |
| Vaginal mucus from pregnant ewes | 32–53 | 2 | 2 | 2 |
| | 108–54 | 2* | 1 | 1 |
| Vaginal mucus from virgin gimmers | 74–55 | 2 | 1 | 1 |
| | 71–55 | 2 | 1 | 1 |
| Fetal fluid from normal parturition | 22–53 | 1 | 0 | 0 |
| | 229–50 | 2 | 2 | 1 |
| Semen | M | 2 | 1 | 1 |
| Control group | Sterile medium | 4 | 0 | 0 |

* One of the guinea pigs died after inoculation; no vibrios were isolated.

collected. In all the other cases, vibrios were isolated from the aborted fetuses, and all showed the same reactions to biochemical testing as the primary strains isolated from ovine fetuses. In one case, the vibrios isolated from the guinea pig fetuses (180–49) had greater catalase activity than the primary strain (7 and 16 mm.).

The abortifacient effect on the guinea pigs of the various Vibrio strains in this material was similar.

*Serological Examinations of the Vibrio Strains Isolated.*—The results of the serological investigations are summarized (table 3).

By titrating the antiserums with the homologous antigen, one serum gave complete agglutination in the dilution 1:5,120 and the other in 1:10,240 or more. Cross agglutinations between each individual serum and all the antigens showed titers indicating the strains belonging to a group with common antigenic properties.

All the agglutination tests with these antigens on *Vibrio fetus*-positive bovine vaginal mucus were negative.

## DISCUSSION

The vibrios isolated from aborting ewes described here are considered to be the abortifacients, as the organisms were found in large numbers and often in pure culture in the aborted fetuses. No other bacteria were found which could have caused the abortions. In addition, vibrios were isolated from the vaginal mucus of pregnant ewes and virgin gimmers, from fetal fluids of normally lambing ewes, from the gastric contents of normally newborn lambs, and from the semen of rams.

In previous years, there has not been an abnormally high number of abortions in the flock, nor has there been any special difficulty with repeat breeding. Therefore, the disease in this flock has the characteristics of a new infection.

All strains tested biochemically reacted similarly with respect to catalase activity, $H_2S$ production, nitrate reduction, and stab culture. In addition, they had the same abortifacient effect on pregnant guinea pigs. Cross-agglutination tests indicate that there are serological relationships between them.

The biochemical tests showed these vibrios to be similar to bovine *Vibrio fetus* except for a much lower catalase activity (under 5 mm.). Older cultures showed a slight increase in catalase activity. One strain showed a great increase in catalase activity after passage through guinea pigs. Some Vibrio strains isolated in late autumn of 1956 from the same herd, and from some of the same animals, showed catalase activity up to 35 mm.

The finding of vibrios in the vaginas of unmated gimmers indicates that a noncoital route of infection is possible. In addition, clinical observations in this herd reveal that infection can be spread among animals in advanced pregnancy after abortions have commenced in a flock. Therefore, it appears important that on the occurrence of Vibrio abortion in ewes, measures for isolation and disinfection be taken as soon as possible to reduce the possibility of infection.

The percentage of abortions in a newly Vibrio-infected flock is given as 30 to 35.[1,2,18] We have observed the same in other flocks where we have detected Vibrio abortion. In this flock, only 12 per cent of the ewes aborted, possibly because of the relatively rapid removal of fetuses and

TABLE 3—Cross-Agglutination Tests on Vibrio Strains Isolated from Sheep

| O + H antiserum / O + H antigen | 149–53 Aborted fetus | 32–53 Pregnant ewe | 74–55 Unmated gimmer | 229–50 Normal parturition | M semen |
|---|---|---|---|---|---|
| 149–53 Aborted fetus | 20,480* | 20,480 | 2,560 | 5,120 | 1,280 |
| 32–53 Pregnant ewe | 10,240 | 20,480 | 320 | 5,120 | 1,280 |
| 74–55 Unmated gimmer | 10,240 | 2,560 | 10,240 | 1,280 | 160 |
| 229–50 Normal parturition | 10,240 | 10,240 | 640 | 10,240 | 640 |
| M Semen | 160 | 160 | 1,280 | 1,280 | 5,120 |

* The figures correspond to the highest dilution giving complete agglutination.

fetal membranes, and the early isolation of aborting ewes.

The fact that vibrios were found at normal parturitions and in vaginal mucus from pregnant ewes which later lambed normally, indicates that not every case of Vibrio infection in ewes need show clinical signs of infection. Abortions occurred toward the end of pregnancy but there was no abnormally high incidence of repeat breeding.

The cattle herd at the farm has been in close contact with the Vibrio-infected flock and their fertility has been normal.

## Summary and Conclusions

1) In a flock of 142 breeding ewes, 18 aborted between gestation days 90 and 140. Lambs aborted before the 110th day of gestation were dead; those aborted later were alive at birth, but died shortly thereafter. Vibrios were isolated from all aborted lambs, with one exception.

2) From animals in the same flock showing no clinical signs of vibriosis, vibrios were isolated from: *(a)* vaginal mucus from pregnant ewes which produced healthy lambs; *(b)* vaginal mucus from virgin gimmers; *(c)* fetal fluid from normally lambing ewes; *(d)* gastric contents from normal lambs at birth; and *(e)* semen from the rams.

3) All of these strains of Vibrio reacted similarly to: *(a)* catalase activity, *(b)* $H_2S$ production, *(c)* nitrate reduction and, *(d)* stab culture tests. The abortifacient effect on pregnant guinea pigs was similar for all strains. Cross-agglutination tests indicated serological relationships between the strains.

4) The investigations revealed that a noncoital route of infection was more important than a coital one. The infection could be spread among animals in advanced pregnancy after abortions commenced in a flock.

5) The isolation of vibrios from vaginal mucus of pregnant ewes which later lambed normally, from the gastric contents of newborn lambs, and from fetal fluid taken at normal parturitions, indicates that not every Vibrio-infected ewe need show clinical signs.

6) Clinically, the infection was characterized by abortion in the later part of pregnancy; repeat breeding was not observed.

7) The cattle herd, in contact with the Vibrio-infected sheep, showed no signs of the infection.

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# The Hair Follicle, Epidermis, Dermis, and Skin Glands of the Dog

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THE COMPOUND nature of the canine hair follicle has been known since 1835 when Gurlt[8] presented his concept of a tuft of hairs emerging from a common orifice. Since that time, many authors have reported variable numbers of hairs in the canine hair follicle.

Claushen[3] states that the hairs in canine skin occur in groups, and that each group usually consists of one main hair and three to nine accessory hairs, all of which arise out of a common hair bed, with the supplemental hairs surrounding the main hair in a half circle. Fuchs[6] found that canine hairs lie in contiguous groups, each with a principal hair and associated hairs; Sisson and Grossman[23] that they are usually arranged in groups of three. Epling[5] has counted as many as 13 hairs sharing a common hair follicle. deMeijere[18] reports that in the family Canidae, hair bundles are arranged in groups of two to four bundles, with groups of three bundles being the most frequent. The typical bundle consists of several wool, or under hairs, and one longer and stiffer guard, or over hair. He also gives evidence which indicates that the number of hairs in a follicle increases with the development of an individual animal. Ellenberger and Baum[4] describe the arrangement of hairs in groups in the dog, and give illustrations of the surface contour of the skin of the dog showing bunches of hairs in groups of three emerging from under folds of the surface. Hietel[11] depicts the folds in the surface of canine skin and arrangements of follicles in groups of three. Hildebrand[12] discusses and describes the surface contour of the skin of the Canidae and suggests that the pattern is similar in the domestic dog.

Sisson and Grossman[23] state that sebaceous glands are best developed in short and rough-haired breeds, and that they are largest and most numerous at the lips, anus, dorsal surface of the trunk, and sternal region; Fuchs,[6] that they occur in elongated and alveolar forms and varying sizes, the largest being on the head, the lip, and the hairy part of the nose; Claushen[3] that they were present in all parts of the skin that he examined; Trautmann and Fiebiger[25] that they are often club-shaped and coiled, or even branched; Webb and Calhoun[26] that they are generally larger than those of man; Nielsen[19] that they are distributed over the entire integument in connection with the hairs. Morphologically, they do not differ from sebaceous glands in the skin of other mammalian species.

Epling[5] describes the histological structure of the sebaceous glands of the dog, their compound arrangement, and association with the compound follicle. Hansen *et al.*[10] state that, at the level of the stratum reticularis, sebaceous glands are bunched on the sides and lower slope of the follicle. They show a complete gradation from germinative to degenerative cells, with little sign of a lumen in any gland. According to Schaffer,[21] all sebaceous glands are developmentally related to hair follicles. Hildebrand[12] describes the modified sebaceous glands on the dorsum of the tail in wild Canidae. Trautmann and Fiebiger[25] describe a supracaudal organ in the cat.

Gurlt[8] was the first to record research on the sweat glands of the dog. He found sweat glands in the hairy skin of the dog that were small, elongated, not very curved, and not easily found. Claushen[3] gives a comprehensive review of the German literature concerning canine sweat glands and includes the observation that they are present in all parts of the skin. Fuchs[6] found them most numerous on the distal part of the limbs, next on the lips, then the hairy part of the nose, and nearly as numerous on the proximal region of the limbs. Speed[24] gives a good survey of the literature and includes histological evidence that the opening of the sweat gland into the hair follicle is separate from that of the sebaceous gland, and closer to the surface of the skin. Aoki and Wada[1] suggest that the sweat glands in the hairy skin of the dog do not participate actively in the central thermoregulatory mechanism, but serve chiefly for the protection of the skin against excessive rise of temperature. Trautmann and Fiebiger[25] state that the apocrine sweat glands of the dog are serpentine, small tubular glands that are better developed in the back, mouth, and anus.

Nielsen[19] classifies the sweat glands of the dog as merocrine and apocrine (according to the classification of Schaffer[21]), and states that the apocrine glands of the dog are loosely coiled sweat glands, distributed over the entire skin as an appendix to the hair follicles, while small, densely coiled merocrine sweat glands are confined to the foot pads. He also included a description, with photomicrographs, of the secreting and resting cycle of the apocrine glands.

This paper presents observations of the morphological features of the hair follicle, epidermis, dermis, surface contour, and glands of the skin of dogs of various ages. It demonstrates the development of the

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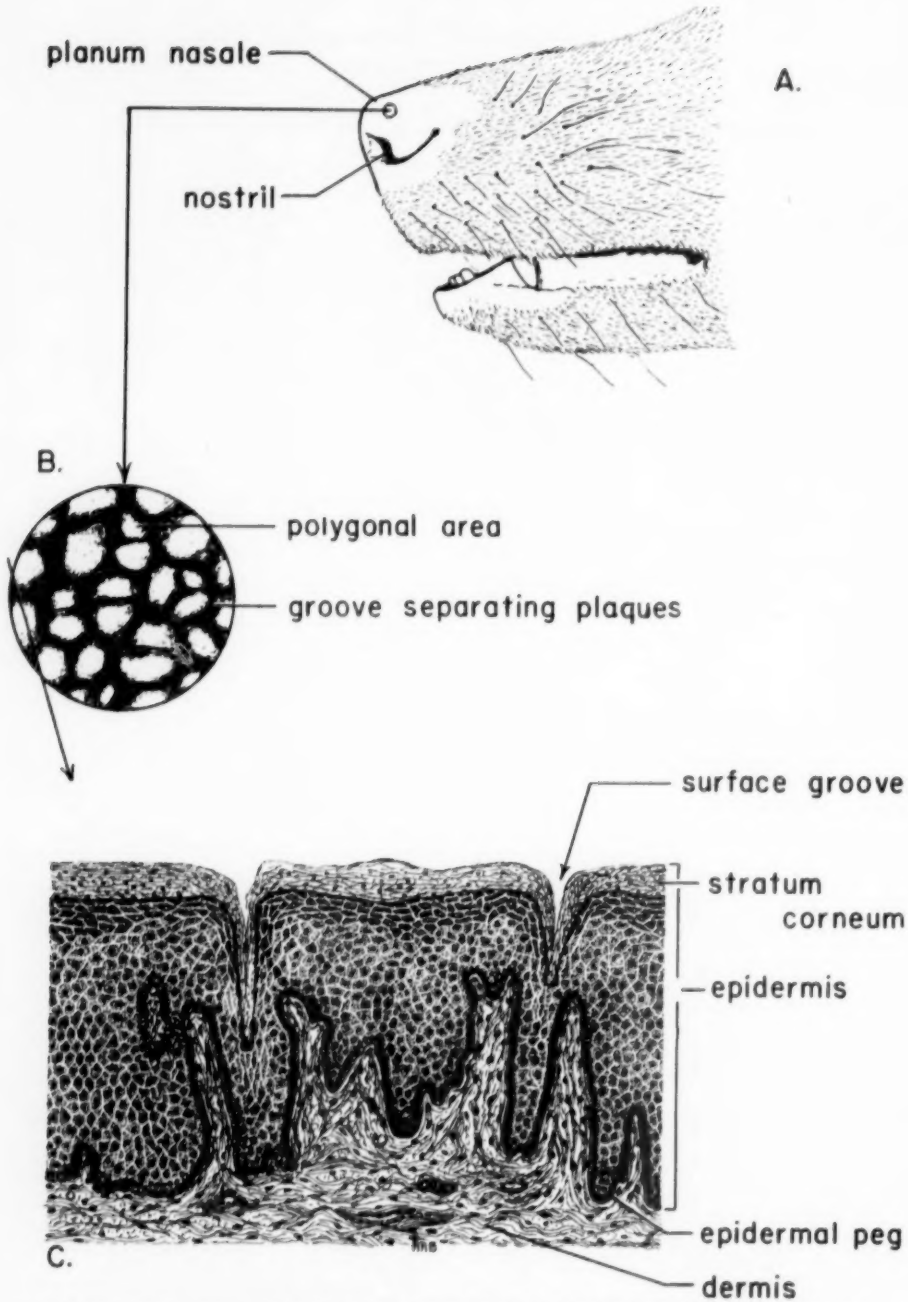


Fig. 1—Surface contour and histology of planum nasale of dog showing: (A) gross appearance of nasal skin; (B) view of polygonal plaquelike areas of planum nasale, x 10; (C) histological section of planum nasale, x 66.

complex follicle, the absence of epidermal pegging (term used by Hansen *et al.*[10]) in hairy canine skin, the presence of epidermal papillae on the skin surface (which have not been previously described), and the existence of specialized sebaceous glands on the dorsal aspect of the tail (similar to those reported in wild Canidae).

## MATERIALS AND METHODS

Observations reported in this paper were made from material prepared for histological and histochemical studies of growth changes of canine skin (Lovell[12]), supplemented by examination of material from embalmed specimens from the veterinary anatomy department, and fresh material from the postmortem laboratory at Iowa State College.

Histological sections were made from the skin of 13 mongrel dogs, ranging from newborn to 28 weeks of age. The dogs represented both the short-haired and long-haired types, and were carefully raised and maintained under as near normal conditions as possible. Skin samples were fixed in 80 per cent alcohol and in 10 per cent formalin, embedded in Altmann's mixture, and serially sectioned at 8 to 12 $\mu$. Formalin-fixed tissue was stained with hematoxylin and eosin, Weigert's, Van Giesen's, Heidenhain's hematoxylin, and Foot's silver impregnation. The tissue fixed in alcohol was treated with the Bauer-Feulgen stain for glycogen, and Gomori's technique for alkaline phosphatase. The results of the histochemical studies will be reported in a future article.

Examination of surface contour, hair arrangement, and tail gland area was made on 34 mongrel dogs which had been embalmed for dissection in the anatomy laboratory, and from 11 dogs which were available (as fresh tissue) shortly after death.

A total of 58 mongrel dogs were used in this study.

For the gross examination of the fresh material with the dissecting microscope, the hair was cut short with electric clippers or scissors prior to the examination.

## OBSERVATIONS

The greatest proportion of the surface of a dog's body is covered by heavily haired skin. A few areas, such as the planum nasale, lips, foot pads, anus, and teats, are devoid of hair. The circumanal glands and anal sacs have been studied by Parks,[20] and Montagna and Parks,[19] so these areas were not investigated.

*The Nasal Skin.*—The nasal skin is usually heavily pigmented, tough, and moist in the living dog. On close examination of the surface of the planum nasale, shallow grooves are observed, which divide the surface into polygonal plaquelike areas, and give the nasal skin a rough appearance (fig. 1B).

Histologically, no glands can be demonstrated (fig. 6). The dermis is composed of reticular, collagenous, and elastic fibers, together with fibroblasts, blood vessels, and nerve trunks. The blood vessels and nerve trunks are larger in the deeper layers of the dermis than in the more superficial layers. The connective tissue fibers are more numerous, with a higher proportion of reticular fibers directly under the epidermis, where the dermis is thrown up into dermal papillae (Ham[9]), or a papillary body (Trautmann and Fiebiger[25]), forming an irregular plane of attachment between dermis and epidermis, which is not parallel with the surface of the skin.

The epidermis, which averages 630 $\mu$ in thickness in adult dogs, is composed of three principal layers: stratum cylindricum, stratum spinosum, and stratum corneum. The stratum cylindricum consists of one layer of cylindrical cells in which mitotic figures frequently are observed. The cells possess little cytoplasm and have dark staining nuclei. The stratum spinosum is made up of 10 to 20 layers of diamond-shaped, dome-shaped, or flattened polygonal cells which have a lighter staining cytoplasm than the cylindrical cells. In heavily pigmented nasal skin, there are many pigment granules in the cytoplasm distal to the nucleus. The more distal spinosum cells apparently do not undergo keratinization as they do in other regions of epidermis. Their cytoplasm becomes weakly acidophilic and the nuclei become pyknotic, with the cells flattening out into a squamous type. As they approach the surface, they remain a thin atypical nucleated stratum corneum, 4 to 8 cell layers thick. Shreds, two to three cell layers in thickness, split off and shed from the surface. The stratum corneum is surprisingly thin for such an apparently tough area (fig. 6).

*The Foot Pads.* The skin of the foot pads is also heavily pigmented and is the toughest region of canine skin. The surface of the pads is rough, due to the presence of numerous conical papillae which are heavily keratinized and readily seen with the naked eye (fig. 2B). When dogs are kept on concrete or rough surfaces, the papillae sometimes become worn so that they are truncated or rounded, instead of conical, in shape.

The interior of the foot pads is made up

of subcutaneous adipose tissue which is partitioned by septa of reticular, collagenous, and elastic fibers (fig. 7). Many elastic fibers are present in the deeper layers. Merocrine sweat glands and lamellar corpuscles are embedded in the adipose tissue of this region.

The dermis is composed of reticular, collagenous, and elastic fibers, fibroblasts, blood vessels, and nerve trunks. The excretory ducts of the merocrine sweat glands traverse the dermis on their way to the surface of the epidermis. Directly under the epidermis, the connective tissue is dense and papillate, forming conical-shaped connective tissue cores for the epidermal conical papillae (fig. 2c). There are also undulations forming secondary dermal papillae within the conical structure (fig. 7).

The epidermis, which averages 1,800 $\mu$ in thickness in adult dogs, is composed of five layers: stratum cylindricum, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum. The stratum cylindricum is made up of a single layer of low columnar basal cells resting on the connective tissue of the dermis. The cells of this layer have dark-staining nuclei which frequently show mitotic activity, and little cytoplasm. The stratum spinosum is

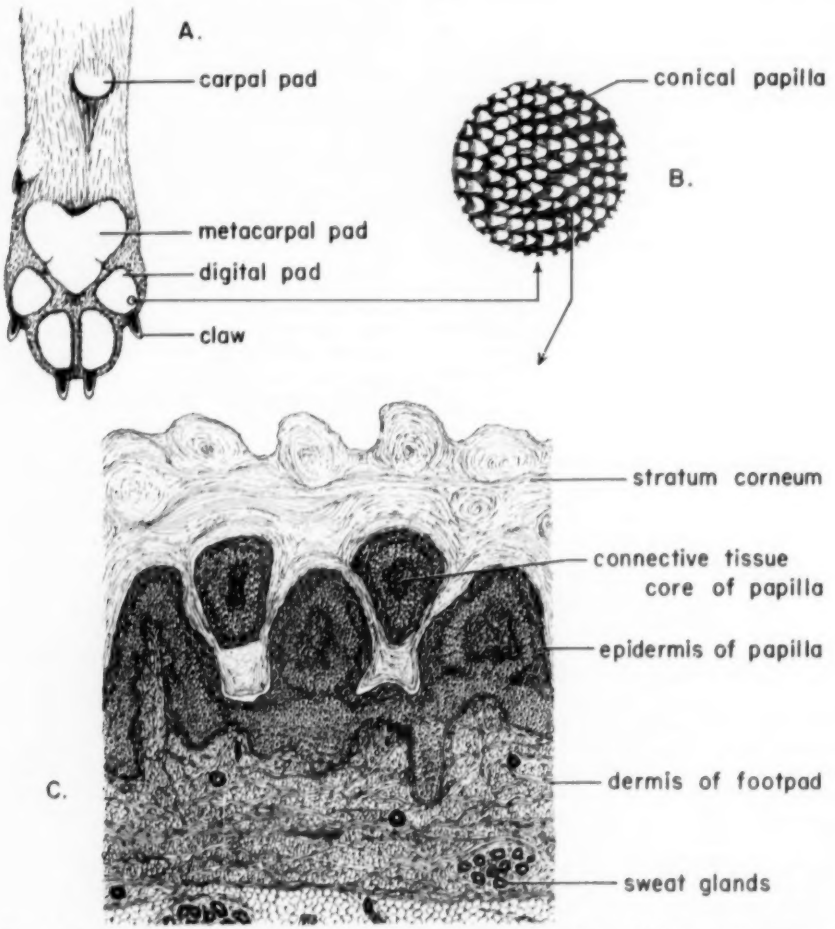


Fig. 2—Surface contour and histology of foot pad of dog showing: (A) gross appearance of foot pads; (B) view of conical papillae arrangement on surface of digital pad, x 8.5; (C) histological section of foot pad, x 57.

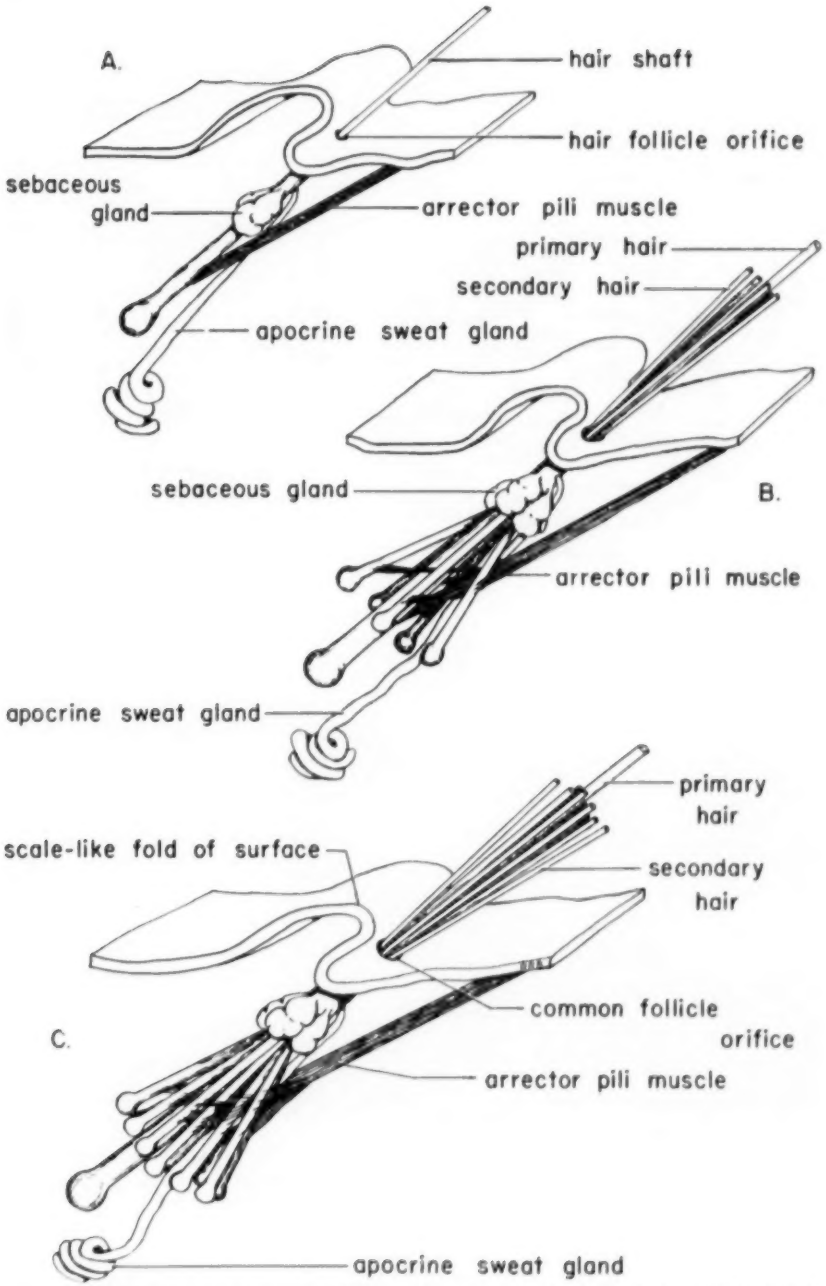


Fig. 3—Development of canine hair follicle: (A) schematic drawing of simple hair follicle as found in the dog during the first week of life; (B) schematic drawing of compound hair follicle as found in the dog during the twelfth week of life; (C) schematic drawing of more elaborate compound hair follicle as found in the dog during the twenty-eighth week of life.

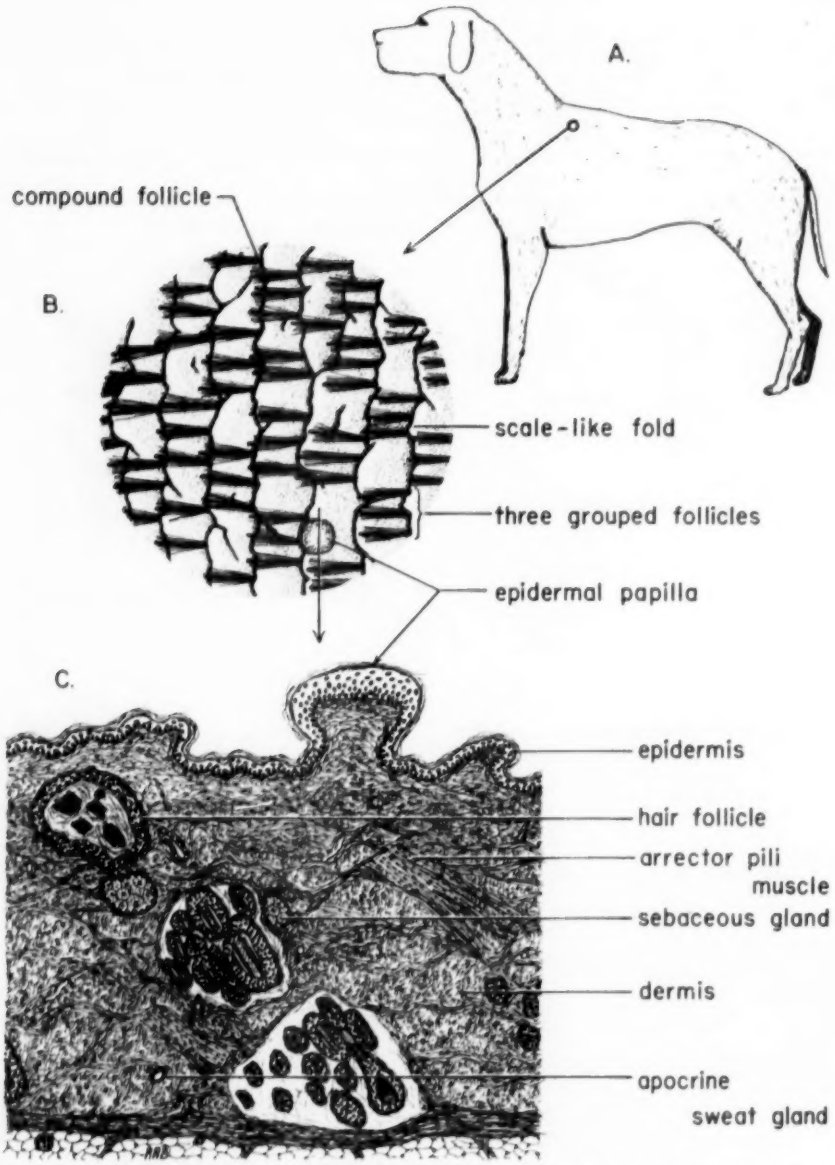


Fig. 4—Surface contour, hair arrangement, and histology of hairy skin of dog: (A) gross appearance of hairy skin; (B) view of scalelike folds and arrangement of hair follicles, x 10; (C) histological section of hairy skin, x 66.

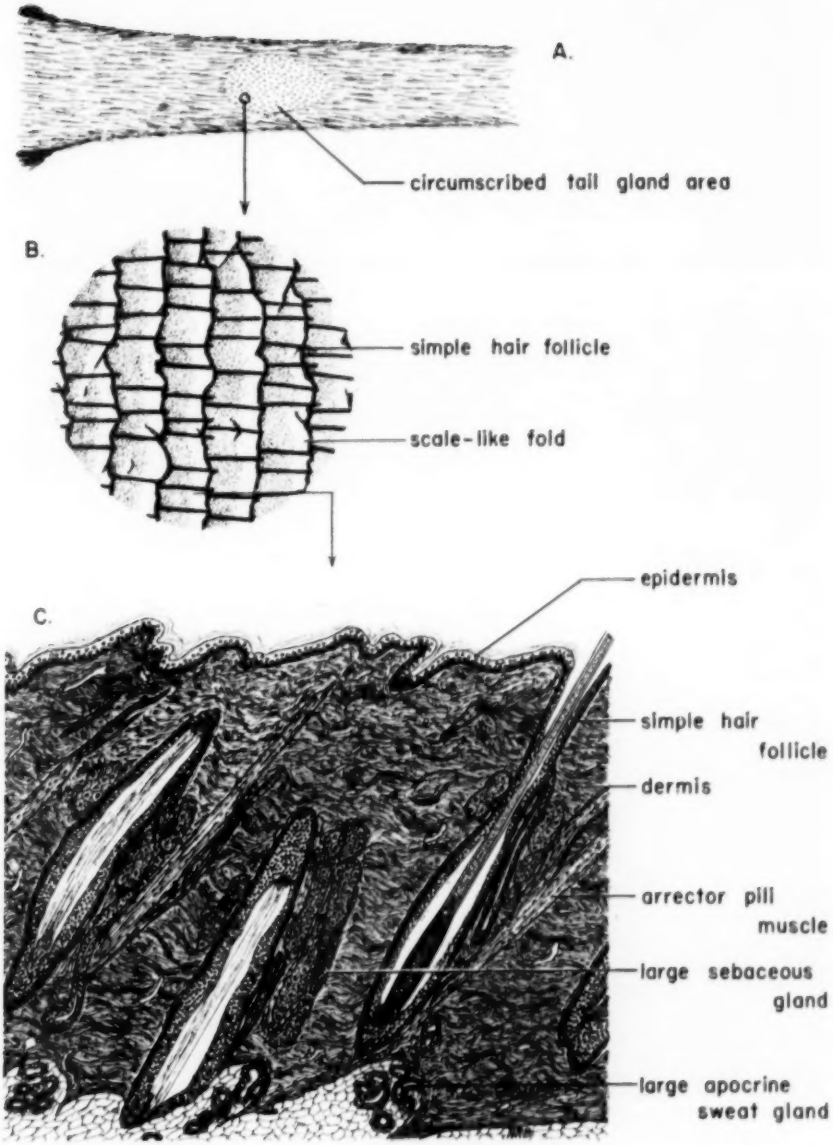


Fig. 5—Surface contour, canine hair arrangement, and histology of supracaudal gland area: (A) gross appearance of tail gland area; (B) view of simple hair follicles in the tail gland area, x 7.5; (C) histological section of tail gland area, x 50.

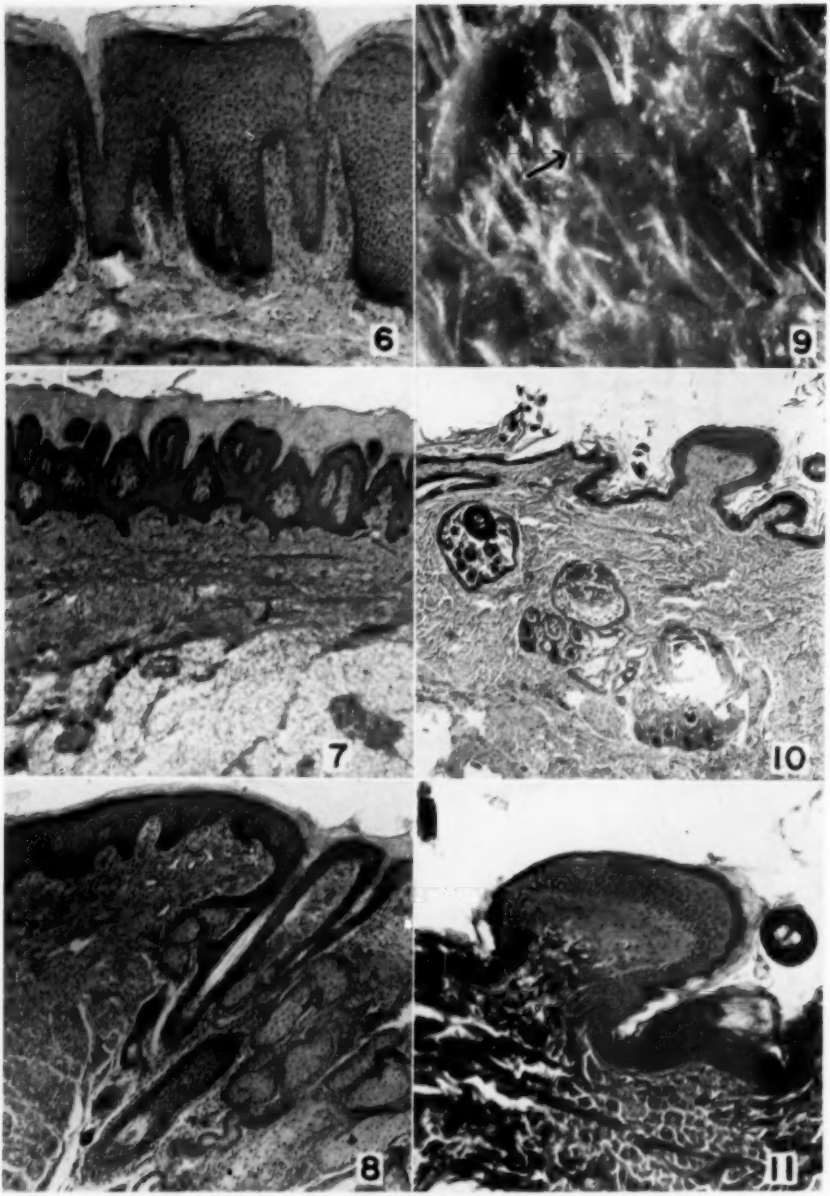


Fig. 6—Nasal skin of dog at 28 weeks—note absence of glands. Section fixed in 10 per cent formalin and stained with Harris' hematoxylin and eosin Y; x 64.

Fig. 7—Foot pad at 8 weeks. Section fixed in 80 per cent alcohol and stained with Harris' hematoxylin and eosin Y; x 25.

Fig. 8—Mucocutaneous junction of lip—note the epidermal pegging of the nonhairy part of the lip. Section fixed in 80 per cent alcohol and stained with Harris' hematoxylin and eosin Y; x 64.

Fig. 9—Epidermal papilla—skin from back region of a mature dog. x 8.5.

Fig. 10—Epidermal papilla—skin section from the interscapular area of a dog 28 weeks of age, showing a typical group of three compound follicles in the dermis. Fixed in 80 per cent alcohol and stained with Harris' hematoxylin and eosin; x 64.

Fig. 11—Epidermal papilla—skin section from the interscapular area of a dog 28 weeks of age. Note the fine connective tissue substance that fills the papilla. Fixed in 80 per cent alcohol and stained with Heidenhain's hematoxylin, Weigert and Van Gieson's connective tissue stain; x 128.

composed of ten to 15 layers of diamond- or dome-shaped cells containing lighter staining nuclei and more cytoplasm than the stratum cylindricum cells. Cell outlines and intercellular bridges may be observed on the spinous cells. The stratum granulosum is made up of four to five layers of flattened cells which contain basophilic keratohyalin granules in their cytoplasm. The stratum lucidum appears as a shiny acidophilic layer of homogeneous substance. The stratum corneum of the foot pads consists of a thick layer of keratinized, nonnucleated material. The stratum corneum is very well developed in the foot pads, where it is usually thicker than all of the cellular layers combined. The excretory ducts of the merocrine sweat glands of the foot pad becomes continuous with the epidermis at the very depths of the epidermal pegs, where their epithelium joins with the stratum cylindricum of the epidermis. The lumen of the excretory duct then follows a tortuous path through the epidermal cells to the surface, where the glandular secretion is expelled.

*Hairy Skin.*—The greatest portion of the surface of canine skin is heavily covered with hairs. There is considerable variation as to type, length, color, direction of slant, and consistency of the individual hair shafts. These features have been studied by several investigators including Gair[7] and Niedoba.[18] A study of the hair follicles of dogs ranging from birth to 28 weeks old, was conducted both grossly and microscopically. Surface examinations were made with a 15 x dissecting microscope and histological sections were studied at 100 x and higher magnifications. Studies of age changes revealed that hair follicles develop from a simple follicle (each follicle containing a single hair) at birth to a compound follicle (each external follicle orifice containing from seven to ten hairs) at 28 weeks of age (fig. 3). The number of hairs per follicle increases concurrently with increases in age. The additional hairs develop in accessory follicles which arise as buds from the original single follicle (fig. 15, 16, and 17). The multiple hairs in the follicle diverge in the deeper layers of the dermis and branch out into separate follicles, in which the hairs originate from individual hair bulbs. The sebaceous glands, which are closely related to the hair follicles, also become compound in nature with an increase in age and empty into the upper portion of the follicle where the hairs are all contained in a single tubular follicle. The arrector pili muscle also becomes compound and branches at its deeper end to send out strands which connect each of the individual follicles in the deeper layers of the dermis (fig. 3c). The apocrine sweat glands remain simple with a single tubular gland sending one excretory duct up along the follicle, which empties into the common part of the follicle complex between the opening of the sebaceous glands and the surface of the skin.

The skin surface is thrown into scalelike folds which form irregular depressions into which the hair follicles invaginate (fig. 4b, 16, and 17). In older dogs which had already developed the compound follicle, each follicle was observed to support one coarse main or guard hair and a number of fine wool, lanugo, or under hairs. The follicles are arranged in groups of two, three, and four with the triad arrangement being the most numerous (fig. 4b).

Small knoblike enlargements, 0.33 to 0.35 mm. in diameter, are present on the surface of the epidermis, projecting from 0.25 to 0.48 mm. above the surrounding area (fig. 9). When these structures were first observed, they were thought to be scars resulting from injuries. However, these enlargements were observed so consistently in different parts of the hairy skin (in dogs of all ages) that it was decided that they must be a regular formation in canine skin. Numerous examinations of skin from embalmed and fresh specimens were performed and one or more of these structures could be demonstrated on each 5 cm.-square piece of skin from any thickly haired portion of the specimens. The appearance of these structures under a dissecting microscope is suggestive of lingual papillae (fig. 4b and 4c). The designation, epidermal papilla, was thought appropriate because of this resemblance.

The hair follicles and apocrine sweat glands extend into the subcutaneous adipose tissue (fig. 17). Above this layer, the dermis consists of a mixture of reticular, collagenous, and elastic fibers, together with fibroblasts, blood vessels, and nerves. The blood vessels and nerves are largest in the deeper layers of the dermis and subcutis. Hair follicles, excretory ducts of the apocrine glands, sebaceous glands, and arrector pili muscles are present in the dermal layer (fig. 17). The connective tis-

sue of the dermis is finer and denser directly under the epidermis than in the deeper layers.

Histologically, the epidermis of the hairy skin, which varies in thickness from 30 to 40 $\mu$, consists of three major layers: stratum cylindricum, stratum spinosum, and stratum corneum. In a few areas, the stratum granulosum and stratum lucidum are evident, but occur only where keratinization is retarded, as around the hair follicle orifices. The total number of layers of cells varies between three and six, but in regions where the stratum granulosum and stratum lucidum are evident, there are as many as eight layers. The stratum cylindricum cells usually have nuclei which are oriented with the long axis perpendicular to the surface of the skin. They have a smaller amount of cytoplasm and stain more intensely than the more superficial cells. Fewer mitotic figures are present in the stratum cylindricum of hairy skin than in the same layer of foot pads and planum nasale. The epidermal papillae are covered by a thickened epidermis which is usually six to 12 cell layers thick, about twice as thick as the surrounding epidermis (fig. 11). The dermis under the epidermal papillae is composed of fine, closely packed connective tissue fibers which are light staining (figs. 10, 11). This fine, dense dermis extends up under the thickened epidermis to form the papilla structure. There is no pegging of the epidermis of the hairy part of the skin (fig. 16). The dermal-epidermal junction of the hairy skin is normally thrown into folds which parallel the surface contour of the skin. This is in contrast to the distinct epidermal pegging present in the epidermis of the planum nasale, the foot pads, and the mucous membranes (fig. 6, 7, and 8).

*Tail Gland Area.*—After the hair has been trimmed short, an area, varying in shape from oval to diamond or arrowhead, may be observed on the dorsal aspect of the tail (3 to 9 cm. from the base of the tail, fig. 5A and 12). In this area, the hairs seem to differ in appearance from the hair of the surrounding area. This has been observed in 45 mongrel dogs, representing both short-haired and long-haired types, and was demonstrated grossly in all cases. When this specialized area of the tail skin is examined under a 15 x dissecting microscope, it is observed that the hairs emerge singly from the hair follicles (fig. 5B). The hair follicles surrounding this area are of the normal complex follicle type supporting six to 11 hairs. The single hairs of the specialized area are coarse, and the surface of the skin has a yellow waxy appearance which is probably due to the abundance of sebaceous secretion. When histological preparations are examined, the simple nature of the hair follicles is apparent (fig. 13 and 14). The sebaceous glands and apocrine sweat glands, which are appendages of the hair follicles, are large, extending deep into the dermis and into the subcutaneous tissue (fig. 13 and 14).

## DISCUSSION

This research failed to give any evidence of glands in the epidermis or dermis of the thick skin of the nose. The continual presence of moisture on the planum nasale could be brought about by the lateral nasal gland, as suggested by Trautmann and Fiebiger,[28] or by serous glands in the nasal passages. The unusually thin stratum cor-

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**Legends for Illustrations on Opposite Page**

**Fig. 12**—Appearance of the circumscribed tail gland area on a mature long-haired dog when the hair is clipped short (arrow indicates tailhead area); 5/7 life size.

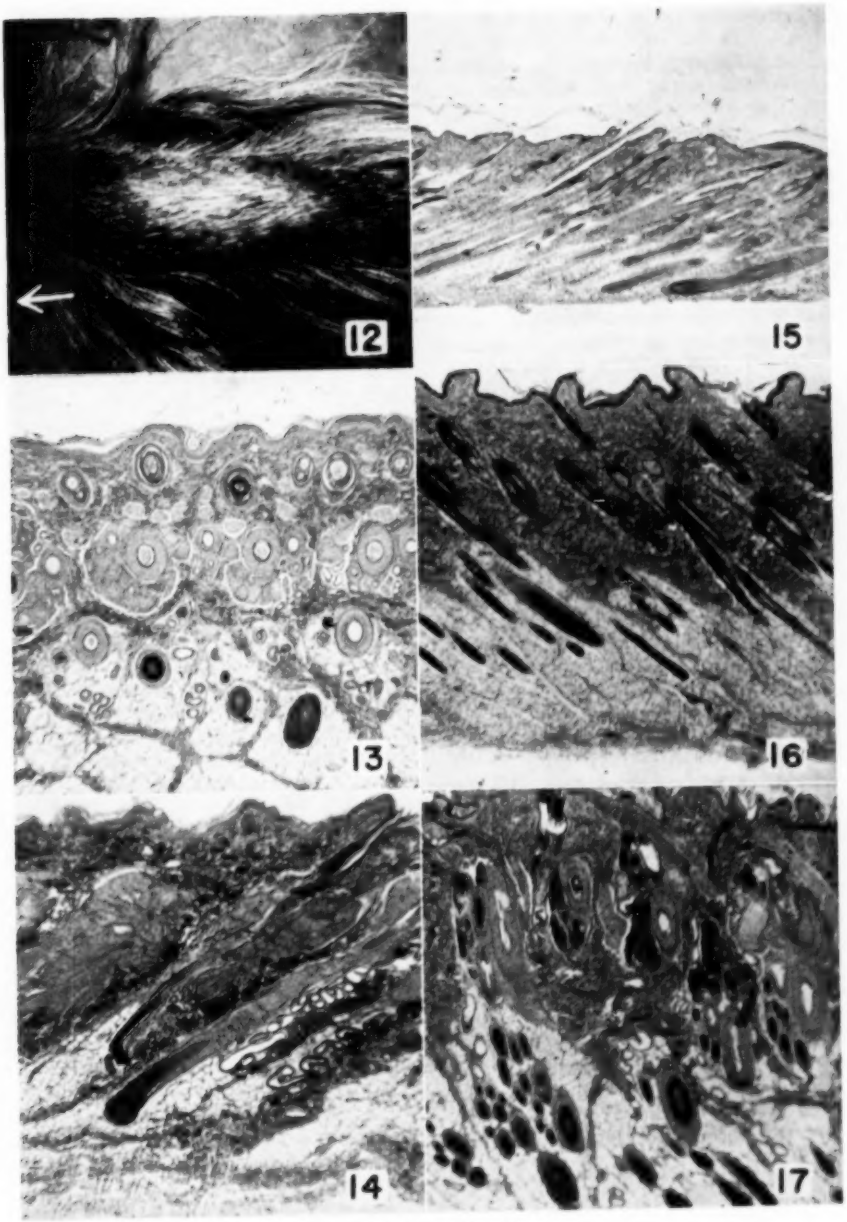
**Fig. 13**—Tail gland area showing large sebaceous glands and apocrine sweat glands (around the cross sections of the single hair follicles). Section fixed in 10 per cent formalin and stained with Harris' hematoxylin and eosin Y; x 25.

**Fig. 14**—Tail gland area—note the large sebaceous glands and apocrine sweat glands beside the longitudinal sections of the single hair follicles. Section from mature dog fixed in 10 per cent formalin and stained with Harris' hematoxylin and eosin Y; x 25.

**Fig. 15**—Hairy skin of dog at birth. Note the absence of compound follicles. Section fixed in 80 per cent alcohol and stained with Harris' hematoxylin and eosin Y; x 25.

**Fig. 16**—Hairy skin of dog at 8 weeks of age, showing how three to four hairs converge at the external follicle orifice. Section fixed in 80 per cent alcohol and stained with hematoxylin and eosin Y; x 25.

**Fig. 17**—Hairy skin of dog at 28 weeks—note that 8 to 10 hairs converge at the external follicle orifice. Section fixed in 80 per cent alcohol and stained with hematoxylin and eosin Y; x 25.




Figures 12 to 17

neum observed on the planum nasale epidermis was atypical in that the cells retained a nucleated condition. There may be some relationship between the continuously moist surface of the nasal skin and the atypical keratinized layer of stratum corneum.

The detailed histology of the foot pads is well described by Epling;[5] however, mention is not made of the stratum lucidum, which was evident in this work. The stratum lucidum in the foot pads was also described by Webb and Calhoun.[26]

In areas of hairy skin, there is no distinct division of the dermis into stratum papillare and reticulare as there is in human skin (Ham[9]), because the dermal-epidermal junction undulates in a line parallel with the surface of the skin. Histological sections show extensive folds and ridges (fig. 4c). As pointed out by Trautmann and Fiebiger,[25] wherever the hair coat is dense, the dermal surface between the hairs is either perfectly smooth or shows only slight elevations and a few small folds.

It would appear that, in the nonhairy surfaces of the skin of dogs, the pegging (rete pegs of Montagna[16]) is present to increase the surface of contact between the epidermis and the dermis for nutritional purposes. This would provide for a maximal area of contact between the vascular and avascular portions. This condition is observed in the food pads, planum nasale, and lip. In heavily haired skin with numerous hair follicles extending down into the dermis, the surface attachment is probably adequate, so that the epidermal pegging is not necessary. The undulations of the surface of the hairy skin would tend to increase the surface somewhat, but not as much as a system of epidermal pegging. Hansen *et al.*[10] report evidence that epidermal pegging develops in the epidermis of hairy skin of dogs which are deficient in dietary fat. Perhaps this is an attempt on the part of the tissue to increase the nutrition of an epidermis which is deficient in some essential elements for normal growth and maintenance.

The surface contour and arrangement of hairs in the skin of domestic Canidae is similar to that of wild Canidae as described by Hildebrand[12] and deMeijere.[15]

Supracaudal glands in the cat have been described by Trautmann and Fiebiger,[25] and in wild Candidae by Hildebrand[12] and Seton.[22] This paper presents evidence that they are also consistently present in domestic Canidae. These specialized gland areas on the dorsal surface of the tail are thought to be related to recognition of the species in wild Canidae. They probably serve the same function in domestic Canidae. The arrector pili muscles of the tail gland area are well developed, as they are along the dorsal aspect of the back.

The observations of this investigation confirm the report of Speed[24] that the ducts of the apocrine sweat glands open into the hair follicles between the sebaceous gland opening and the surface of the skin.

The histological structure of the sebaceous glands was found to agree with the descriptions of Epling,[5] Nielsen,[19] and Hansen *et al.*[10] The histological structure of both the merocrine sweat glands of the foot pads and the apocrine sweat glands of the hairy skin was found to agree with that described by Nielsen.[19]

## SUMMARY AND CONCLUSIONS

The subgross anatomy and histology of the canine hair follicle, epidermis, dermis, and skin glands, excluding the circumanal glands and glands of the anal sacs is described and illustrated on the basis of studies of 58 mongrel dogs. The following observations warrant emphasis:

1) The hair follicle of the dog develops from a simple follicle at birth to a compound follicle at maturity.

2) The compound follicles of dog skin are arranged in groups of two, three, and four, with groups of three being the most common.

3) Epidermal pegging is present in the epidermis of the foot pads, the planum nasale, and the lip.

4) The hairy skin of the dog has no epidermal pegging at the dermal-epidermal junction.

5) Epidermal papillae are normally present on the surface of the epidermis of the hairy skin of the dog.

6) The surface contour of the hairy skin of the dog is made up of ridges and valleys. The hair follicles invaginate into the depths of the valleys.

7) The epidermis of the planum nasale is divided by grooves into polygonal plaque-like structures.

8) The stratum corneum is thin on the planum nasale.

9) The surface of the foot pads is studded

with conical shaped papillae which are highly keratinized.

10) The stratum corneum of the foot pads is thick.

11) Domestic Canidae possess a specialized cutaneous gland area on the dorsal aspect of the tail which is characterized by simple hair follicles supporting single hairs, large sebaceous glands, apocrine sweat glands, and arrector pili muscles.

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# The Effect of Five Proteolytic Enzymes on Some Antigenic Characteristics of Rabies Virus—with Observations on the Resistance of the Virus to Heat

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A METHOD that would liberate greater amounts of the rabies virus from the brain than mechanical maceration alone would facilitate the diagnosis of rabies in cases in which the titer of the virus is low. It is known that the sediment of brain tissue which is precipitated by centrifugation contains a large percentage of the available virus.[6,13] Therefore, additional quantities of active virus might be liberated by proteolysis of macerated infected brain tissue.

The five proteolytic enzymes which were studied produced a 9 to 20 per cent decrease in brain protein,[32] in addition to the amount of degradation accomplished by emulsification. If an equivalent percentage of active rabies virus were released with proteolysis, a significant increase in the titer might result.

The action of various enzymes of animal and vegetable origin on rabies virus has been studied by several investigators, but their work was limited to determining whether a given enzyme inactivated the virus. They made no efforts to ascertain whether the enzymes which did not inactivate the virus had liberated additional quantities of virus. Therefore, this study was undertaken to investigate the effect of a selected group of proteolytic enzymes on the elution of rabies virus.

## REVIEW OF LITERATURE

Remlinger[22] reported in 1905 that a "thick emulsion of fixed rabies virus," when placed in "viscus" sacks and inserted in the peritoneal cavity of dogs or rabbits, was inactivated within 24 hours. After a thorough investigation, he concluded that, "It remains, therefore, to incriminate a possible and singularly energetic rabicide of the liquid of the peritoneal cavity."

In 1934, Hirano[12] studied the effects of lipase, trypsin, and diastase on fixed rabies virus by conducting a series of animal inoculations with various dilutions of virus and enzyme. The virus was unaffected by lipase in a dilution of 1:10,000, partially attenuated by a 1:8,000 dilution, and completely destroyed by a 1:4,000 dilution. When he used a 5 per cent dilution of trypsin in 50 per cent glycerol, diluted with phosphate buffer, the virus resisted the action of trypsin in dilutions greater than 1:100. Results with a 10 per cent solution of diastase in 50 per cent glycerol were similar. In dilutions of 1:40 and greater, diastase had no effect on the virus.

Jonnesco[16] investigated the natural immunity of some animals to rabies in 1934, and in a subsequent publication stated that, "Enzymes appeared to have an important role in the processes of defense against rabies." In 1936, he[15] demonstrated that human pancreatic juice, in maximum dilutions of 1:10, can have a destructive effect on the virus.

Bailly,[1] in 1945, studied the action of pancreatic extract on fixed rabies virus. He concluded that an aqueous extract of dog pancreas at a pH of 10 destroyed the virulence of the virus after 24 hours of contact at 10 C.

In 1947, Remlinger and Bailly[23] subjected a 1:50 brain emulsion from a rabbit infected with fixed virus, to proteolysis with fig latex. The emulsion was mixed with serial dilutions of the latex and incubated for 17 hours at 15 C. Mice inoculated intracerebrally with each dilution survived, but other mice inoculated with an aqueous suspension of the untreated brain emulsion died. The authors cited evidence that the juice of various species of aloes had produced similar destructive effects on rabies virus.

The proteolytic enzyme, cathepsin, is present in autolyzed tissue,[2] and may have some effect on rabies virus. The virus will resist putrefaction for a long period, if protected from other detrimental influences.[10,18] It has been recovered after four months from autolyzed brain kept at 18 C., but after only a few days when kept at 37 C.[26] Johnson[13] stated that "Tissue enzymes liberated from brain material used in the preparation of rabies vaccine undoubtedly have a deleterious effect on the antigenicity of the virus."

## MATERIALS

*Standard Diluent.*—A mixture of 9 parts Krebs-Ringer-phosphate solution, pH 7.6, and 1 part antibiotic solution (12,500 units of penicillin G and 25 mg. of streptomycin sulfate per ml.) was the standard diluent. The Krebs-Ringer solution was prepared in the manner described by Cohen[5] as modified by Graea.[7] To 90 ml.

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of the Krebs-Ringer solution, 10 ml. of a phosphate buffer,[35] pH 7.6, was added.

*Proteolytic Enzymes.*—The five proteolytic enzymes used had been previously determined[31] to be nontoxic to mice when employed in concentrations which would produce significant proteolysis of brain tissue in four hours at 37 C.[32]

Trypsin.*—Trypsin hydrolyzes protein to peptides and amino acids.[11] Pure trypsin gradually digests itself at pH 7.0 to 9.0. Its optimal temperature and pH are 37 to 50 C. at pH 7.8 to 8.7.

Rhozyme P-11.*—This is a fungal protease produced from an aqueous extract of the fungus by alcohol precipitation.[20, 33] The manufacturer did not reveal the identity of the fungus. The optimal temperature for its proteolytic activity is 50 C., but satisfactory results are obtained at lower temperatures. The optimal pH range is 6.5 to 7.5.

Protease 15.*—This is a bacterial protease prepared much as is Rhozyme P-11.[20, 34] Its action lowers the viscosity of protein solutions and increases the solubility of the protein but does not proceed as far as amino acids. The optimal temperature is 50 C., but satisfactory results may be obtained at lower temperatures. The optimal pH is 6.5 to 7.5, with a working range between pH 5.5 to 8.5.

Protease (trypsin).*—The optimal conditions for the action of the two components of this enzyme (protease and peptidase) are different.[20] The optimal pH for its proteolytic activity is 7.5 to 8.5 at a temperature of between 40 and 52 C.

Proteinase A.*—This is a crude preparation made from the filtrates of a culture of an aerobic soil saprophyte.[20, 29] The optimal temperature and pH are 37 C. at pH 7.0.

*Experimental Animals.*—Adult albino rabbits, 10 to 15 lb., were used as indicated. They were procured from a local source and their strain was unknown.

Two strains, S-P and CFW, of albino Swiss mice, weighing 20 Gm., were used without preference as to the strain or sex. Their age and weight had no discernible influence on the experimental results obtained.[4, 17]

*Viruses.*—Two strains† of rabies virus were employed as control viruses. One virus, the National Institutes of Health standard challenge virus No. 59 (CVS), was a fixed virus. The other was Lederle R174BK222AP99 (7-20-54) street virus (NY).

Three strains of street rabies virus, isolated from dogs, were obtained from the Virology Section, Fourth Army Area Medical Laboratory, Fort Sam Houston, Texas. They will be referred to by the case number originally assigned and the prefix FAAML.

All five strains of virus were identified as rabies by means of serum-virus neutralization tests.[19] Filtered (Seitz E-K), undiluted equine serum was employed as the normal serum. Lederle's concentrated anti-rabies serum, dialyzed, filtered (Seitz E-K), and diluted 1:10 with physiological salt solution was employed as the immune serum.[5, 18]

* Trypsin supplied by Gen. Bio-Chemicals Inc., Rhozyme P-11 and Protease 15 supplied by Rohm & Haas Co., Protease (trypsin) supplied by Takamine Laboratory, and Proteinase A by Sharp & Dohme.

† Both strains were furnished by the Fort Dodge Laboratories.

## METHODS

*Rabies Virus Elution in the Presence of Five Proteolytic Enzymes.*—Each of the canine brains from which a strain of FAAML virus was isolated was emulsified in a chilled Waring blendor and diluted to a 40 per cent suspension by weight. The CVS and NY strains of challenge virus were each diluted by volume to a 10 per cent suspension. The five suspensions were centrifuged for 15 minutes at 1,200 x g, and 0.1 ml. of each supernatant fluid was inoculated intracerebrally into rabbits. All inoculations were performed under deep ether anesthesia. Brain impression smears from each rabbit that died subsequent to inoculation, were prepared and examined microscopically for Negri bodies.[26, 28] Each of the rabbit brains containing the CVS, NY, and FAAML strains of virus, was emulsified in a sterile, chilled Waring blendor and 1 Gm. of each was used to prepare a 20 per cent suspension, by weight, in physiological salt solution. These suspensions were subjected to serum-virus neutralization tests.

Standard solutions of the five proteolytic enzymes were prepared. Each contained the following amount, by weight, of enzyme per 9 ml. of diluent: trypsin 0.125 Gm.; Rhozyme P-11 0.1 Gm.; Protease 15 0.125 Gm.; Protease (trypsin) 0.125 Gm.; and Proteinase A 2,500 units. Ten per cent suspensions, by weight, of each virus were prepared with each enzyme standard dilution. Similar suspensions with standard diluent were designated as 4 C. and 37 C. controls.

The 4 C. controls for each of the stock virus suspensions were held at that temperature for four hours. The 37 C. controls and the suspensions of each stock virus in the standard solutions of enzymes, were incubated for four hours at 37 C. (one hour in a water bath and three hours in a roller-tube drum in a dry heat incubator). All suspensions were then centrifuged for 15 minutes at 1,200 x g. Serial tenfold dilutions, $10^{-1}$ through $10^{-5}$ were prepared from each supernatant fluid.

For the infectivity titrations of the virus emulsions subjected to proteolysis, 4 mice were inoculated intracerebrally[17] with 0.03 ml. of each serial dilution, and observed for 21 days. A total of 700 mice were used. Deaths occurring more than five days after inoculation were considered to be due to rabies. Mice which survived, but exhibited signs on the last day, were considered to be infected with rabies. The $l.d._{50}$ end point of each titration was estimated by the method of Reed and Muench.[21]

*Challenge of Mice Which Survived in the Titration of Serial Dilutions of Stock Virus-Brain Emulsions Subjected to Proteolytic Enzymatic Digestion.*—The brain emulsions from the first

rabbit passage of each stock virus were employed as challenge viruses. The challenge virus suspensions were diluted to contain 4 $l.d._{50}$ per 0.03 ml. of inoculum, based on the $l.d._{50}$ of the 4 C. controls.

At the conclusion of the observation period, all surviving mice were challenged by intracerebral inoculation of 4 $l.d._{50}$ of the homologous challenge virus suspension. Six normal mice were also inoculated with each challenge virus.

*Attempts to Immunize Mice Against Rabies by a Single Inoculation of a Supernatant Fluid of* CVS *Rabbit Brain Emulsion Subjected to Proteolytic Enzymatic Digestion.*—Two rabbits were inoculated intracerebrally with 0.25 ml. of a 20 per cent suspension of brain emulsion from the first rabbit passage of the CVS strain of virus. They were killed on the seventh day after inoculation and their brains were harvested and emulsified. Two, 2 Gm. portions of the brain emulsion were diluted to a 20 per cent suspension, by weight, with Rhozyme P-11 standard solution containing 0.2 Gm. of enzyme per 8 ml. of diluent. These suspensions were incubated for four hours at 50 C. and both suspensions were centrifuged for 15 minutes at 1,200 x g. The supernatant fluids of each were pooled, diluted to 10 per cent and labeled Rhozyme P-11 vaccine No. 1.

The same procedures were repeated with the modification that the enzyme standard solution contained 0.25 Gm. of Protease 15 in place of Rhozyme P-11. The resultant vaccine was labeled Protease 15 vaccine No. 1.

Both experimental vaccines were titrated for viability by intracerebral inoculation of mice with serial tenfold dilutions, $10^{-4}$ through $10^{-9}$. Five mice received 0.03 ml. of inoculum per dilution and were observed for 14 days.

Serial tenfold dilutions, $10^{-4}$ through $10^{-9}$, were prepared from the brain emulsion of the second rabbit passage of CVS virus. Six mice were inoculated intracerebrally with 0.03 ml. of inoculum per serial dilution and were observed for 14 days. The number of $l.d._{50}$ in 0.03 ml. of undiluted rabbit brain emulsion was expressed antilogarithmically.

Upon the completion of the viability tests, 30 mice were inoculated intraperitoneally with a single 0.5 ml. dose of experimental vaccine, and observed for 14 days.

A 10 per cent suspension of phenolized rabies vaccine of caprine origin (Pitman-Moore, No. 692656) was employed as the control vaccine; 30 mice were inoculated intraperitoneally with 0.5 ml., and observed for 14 days.

Based on the number of $l.d._{50}$ in 0.03 ml. of undiluted rabbit brain emulsion of the second passage of CVS virus, serial dilutions were prepared to contain approximately 12,400; 9,300; 6,200; 2,100; and 120 $l.d._{50}$ per 0.03 ml. On postvaccination day 14, each group of 30 mice inoculated with experimental and control vaccine was divided into six lots of 5 each. Mice from one lot from each group were inoculated intracerebrally with 0.03 ml. of inoculum per serial dilution of challenge virus, and observed for 14 days. Deaths occurring after the fifth day following inoculation were considered to be due to rabies. A group of 30 unvaccinated mice was similarly inoculated to serve as controls.

*Attempts to Immunize Mice Against Rabies by Multiple Inoculations of Vaccines Produced from* CVS *Rabbit Brain Emulsion Subjected to Proteolytic Digestion.*—Six rabbits were inoculated intracerebrally with 0.25 ml. of a 20 per cent suspension of brain emulsion from the second rabbit passage of the CVS virus. The rabbits were killed on the seventh day after inoculation, and their brains emulsified to form a pool of stock emulsion. Serial tenfold dilutions of a 10 per cent suspension of this brain emulsion were titrated by intracerebral inoculation into mice. The number of $l.d._{50}$ in 0.03 ml. of undiluted emulsion was expressed antilogarithmically.

Sixteen, 1 Gm. portions of the brain emulsion were diluted to 10 per cent suspensions, by weight, with a solution containing 0.1 Gm. of Rhozyme P-11 per 9 ml. of diluent. These suspensions were incubated for one hour at 50 C. in a water bath followed by three hours at 37 C. in a roller-tube drum, in a dry heat incubator. At the end of the incubation period, eight of the suspensions were identified as Rhozyme P-11 vaccine suspension No. 2. The remaining suspensions were centrifuged for 15 minutes at 1,200 x g, and the supernatant fluids were pooled and identified as Rhozyme P-11 vaccine supernatant fluid No. 2. The procedure was repeated employing a solution containing 0.125 Gm. of Protease 15 per 9 ml. of diluent.

Sixteen, 1 Gm. portions of brain emulsion were diluted to 10 per cent suspensions, by weight. The same procedures were used to produce heat inactivated CVS vaccine suspension, and vaccine supernatant fluid. A 10 per cent suspension of phenolized rabies vaccine, of caprine origin (Pitman-Moore, No. 692,665), was designated as the control vaccine suspension. An equal quantity of phenolized vaccine was centrifuged for 15 minutes at 1,200 x g, and the resultant supernatant fluid designated as control vaccine supernatant fluid.

The six vaccine preparations were titrated for viability by intracerebral inoculation in mice. The brain of each mouse that died on or after postinoculation day 5 was harvested. After 14 days of observation, each pool of mouse brains was emulsified. A 20 per cent suspension of each emulsion was diluted to 1:360 so as to contain an estimated 100 $l.d._{50}$ per 0.03 ml., and was subjected to the modified serum-virus neutralization test.[10]

Separate groups of 20 mice each were inoculated with the vaccine preparations. The schedules of the modified Habel test for potency were used.[9]

A 1:79 suspension of brain emulsion from the second rabbit passage of the CVS virus, which contained 500 $l.d._{50}$ per 0.03 ml., was used as the challenge virus.

## RESULTS

*Rabies Virus Elution in the Presence of Five Proteolytic Enzymes.*—Each virus suspension subjected to 37 C. for four hours, exhibited a marked decrease in viral

**TABLE 1—Effects of Five Proteolytic Enzymes on Five Strains of Rabies Virus**

| Virus | Controls | | Decrease in viral activity (%) | Test preparations | | | | | Approx. (%) decrease in viral activity due to enzymes |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Proteolysis at 37 C. with | | | | | |
| | 4 C. | 37 C. | | Trypsin | Rhozyme P-11 | Protease 15 | Protease (tryp.) | Proteinase A | |
| CVS | 100,000* | 19,900 | 80 | 10 | 30 | 199 | 15 | 10 | 19 |
| NY | 3,160 | 1,000 | 66 | 100 | 19 | 30 | 10 | 19 | 33 |
| FAAML | | | | | | | | | |
| 309 | 10,000 | 1,990 | 80 | <10 | <10 | 10 | 30 | 10 | 19 |
| 363 | 316 | 199 | 37 | <10 | <10 | <10 | <10 | <10 | >60 |
| 372 | 50 | 19 | 60 | <10 | <10 | <10 | 15 | <10 | 26 |

* Number of residual $l.d._{50}$ of active virus in 0.03 ml. of brain emulsion after four hours' incubation.

activity (table 1). The decrease varied from 37 to 80 per cent among strains of the virus. Each strain possessed an individual level of heat resistance which exhibited some relation to its original titer. The CVS, NY, and FAAML 309 strains, which possessed a relatively high titer before heat treatment, appeared to be proportionately less resistant than those of low initial titer.

A decrease in viral activity was noted for each virus treated by any one of the five enzymes (table 1). These decreases varied for each strain of virus and exceeded the decreases of viral activity attributed to 37 C. for four hours. The results indicate that the virulence of each strain of virus tested was attenuated by subjecting the virus to proteolysis with trypsin, Rhozyme P-11, Protease 15, Protease (trypsin), or Proteinase A for four hours at 37 C.

*Challenge of Mice Which Survived in the Titration of Serial Dilutions of Stock Virus Brain Emulsions Subjected to Proteolytic Enzymatic Digestion.*—A sufficient number of mice survived the challange inoculation of 4 $l.d._{50}$ of homologous virus to indicate that some degree of immunity had been established, which was associated with the amount of inactive virus in each control and enzyme-brain emulsion titration. This can be seen by comparing the results shown in tables 1 and 2. The decrease in active virus in the control and enzyme-brain emulsion preparations appeared to be related to the survival rate of the mice following challenge. The CVS, NY, and FAAML 309 viruses each exhibited a decrease of 3,000 $l.d._{50}$ or more in the original titrations (table 1). The degree of the immunity exhibited against these viruses (table 2) appeared to be proportional to the amount of inactive virus in the original titrations. Such a relation did not exist for those viruses in which the decrease of $l.d._{50}$ was less than 3,000 in the original titrations. Rhozyme P-11 and Protease 15 enzyme-brain emulsion preparations most consistently established an immunity against 4 $l.d._{50}$ of homologous virus.

*Attempts to Immunize Mice Against Rabies by a Single Inoculation of a Supernatant Fluid of* CVS *Rabbit Brain Emulsion Subjected to Proteolytic Enzymatic Digestion.*—The second rabbit passage of the CVS virus contained 39,820 $l.d._{50}$ per 0.03 ml. of the pooled brain emulsion. In the titration of Rhozyme P-11 vaccine No. 1, and Protease 15 vaccine No. 1, no deaths occurred among mice of any of the groups. Detectable residual active virus was not present in either vaccine.

No deaths occurred among any of the groups of mice which were inoculated intraperitoneally with Rhozyme P-11 vaccine No. 1, Protease 15 vaccine No. 1, or the phenolized control vaccine. All the mice which had been vaccinated with a single 0.5 ml. dose of the two experimental vaccines and the controls died subsequent to challenge with serial dilutions of homologous virus. In the group of mice which received phenolized vaccine, the survival rates were extremely poor. Of these controls, only 1 mouse out of 5 survived in the groups inoculated with serial dilutions of challenge virus 120 $l.d._{50}$ through 6,200 $l.d._{50}$ per dose, and none which received the higher concentrations of challenge virus survived.

*Attempts to Immunize Mice Against Rabies by Multiple Inoculations of Vaccines Produced from* CVS *Rabbit Brain Emulsion Subjected to Proteolytic Enzymatic Digestion.*—The third rabbit passage of the CVS virus contained 39,820 $l.d._{50}$ per 0.03 ml. of the pooled brain emulsion. Deaths occurred only among the groups of mice given intracerebral inoculations of

$10^{-1}$ dilutions of Rhozyme P-11 vaccine suspension and supernatant fluid No. 2, Protease 15 vaccine suspension and supernatant fluid No. 2, and the heat-inactivated control vaccine suspension and supernatant fluid (table 3). In the case of each vaccine suspension and supernatant fluid, exposure to 50 C. for one hour, followed by 37 C. for three hours, was insufficient to completely inactivate the CVS virus as shown by the presence of residual active virus.

By comparing the residual $l.d._{50}$ of active virus with the 39,820 $l.d._{50}$ for the untreated brain emulsion from which the vaccines were prepared, it is evident that Rhozyme P-11 and Protease 15, plus heat, inactivated a minimum of 39,800 $l.d._{50}$ of virus per 0.03 ml. of brain emulsion. A similar comparison with the titers of the heat-inactivated vaccines indicates that the heat treatment alone inactivated a minimum of 39,790 $l.d._{50}$ of virus. The effect of the heat treatment employed in the preparation of the vaccines accounted for over 99 per cent of the total amount of virus inactivated. The results of the serum-virus neutralization tests, performed with brain material harvested from the vaccine viability titrations, indicated that the vaccines did contain active virus.

No deaths occurred among the groups of

**TABLE 2—Results of the Challenge of Mice Which Survived in the Titrations of Rabies Virus Subjected to Proteolysis**

| Virus strain and $l.d._{50}$ titer of 4 C. control | Challenge dilution of virus represented by 4 $l.d._{50}$ in 0.03 ml. | Original test preparation | Mortality ratios of mice | | | | |
|---|---|---|---|---|---|---|---|
| | | | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ |
| CVS | 1:25,000 | Challenge control | 6/6 | ..... | ..... | ..... | ..... |
| | | 4 C. control | ..... | ..... | ..... | ..... | 0/2 |
| 100,000 | | 37 C. control | ..... | ..... | ..... | 0/1 | 0/4 |
| | | Trypsin | ..... | 0/4 | 0/4 | 1/4 | 1/4 |
| | | Rhozyme P-11 | ..... | 0/2 | 0/4 | 0/4 | 0/4 |
| | | Protease 15 | ..... | 0/1 | 0/4 | 0/4 | 0/4 |
| | | Protease (trypsin) | 2/2 | 0/3 | 0/4 | 1/4 | 1/4 |
| | | Proteinase A | ..... | 1/4 | 0/4 | 2/4 | 2/4 |
| N Y | 1:790 | Challenge control | 6/6 | ..... | ..... | ..... | ..... |
| | | 4 C. control | ..... | ..... | ..... | 3/4 | 2/4 |
| 3,160 | | 37 C. control | ..... | ..... | 2/2 | 2/4 | 2/4 |
| | | Trypsin | ..... | 1/2 | 3/4 | 3/4 | 2/4 |
| | | Rhozyme P-11 | 0/1 | 1/4 | 1/4 | 0/4 | 0/4 |
| | | Protease 15 | 1/1 | 2/3 | 1/4 | 0/4 | 0/4 |
| | | Protease (trypsin) | ..... | 2/4 | 3/4 | 1/4 | 1/4 |
| | | Proteinase A | 0/1 | 2/4 | 3/4 | 3/4 | 2/4 |
| FAAML 309 | 1:2,500 | Challenge control | 6/6 | ..... | ..... | ..... | ..... |
| | | 4 C. control | ..... | ..... | ..... | 2/2 | 4/4 |
| 10,000 | " | 37 C. control | ..... | ..... | 1/1 | 4/4 | 4/4 |
| | | Trypsin | 0/4 | 3/4 | 2/4 | 4/4 | 2/4 |
| | | Rhozyme P-11 | 1/4 | 1/4 | 0/4 | 0/4 | 0/4 |
| | | Protease 15 | 2/2 | 1/4 | 2/4 | 0/4 | 0/4 |
| | | Protease (trypsin) | ..... | 1/4 | 1/4 | 1/4 | 0/4 |
| | | Proteinase A | 2/2 | 0/4 | 0/4 | 0/4 | 0/4 |
| FAAML 363 | 1:79 | Challenge control | 6/6 | ..... | ..... | ..... | ..... |
| | | 4 C. control | ..... | 0/1 | 3/3 | 4/4 | 4/4 |
| 316 | | 37 C. control | ..... | 0/1 | 4/4 | 4/4 | 4/4 |
| | | Trypsin | 4/4 | 4/4 | 4/4 | 4/4 | 4/4 |
| | | Rhozyme P-11 | 4/4 | 4/4 | 4/4 | 4/4 | 4/4 |
| | | Protease 15 | 4/4 | 3/4 | 4/4 | 4/4 | 4/4 |
| | | Protease (trypsin) | 4/4 | 4/4 | 4/4 | 4/4 | 3/4 |
| | | Proteinase A | 4/4 | 4/4 | 4/4 | 4/4 | 4/4 |
| FAAML 372 | 1:12 | Challenge control | 6/6 | ..... | ..... | ..... | ..... |
| | | 4 C. control | 0/1 | 0/2 | 0/4 | 2/4 | 2/4 |
| 50 | | 37 C. control | 0/1 | 0/4 | 1/4 | 2/4 | 2/4 |
| | | Trypsin | 1/4 | 1/4 | 3/4 | 3/4 | 1/4 |
| | | Rhozyme P-11 | 0/4 | 0/4 | 3/4 | 2/4 | 3/4 |
| | | Protease 15 | 2/4 | 2/4 | 3/4 | 0/4 | 1/4 |
| | | Protease (trypsin) | 1/2 | 2/3 | 2/4 | 2/4 | 1/4 |
| | | Proteinase A | 2/4 | 2/4 | 0/4 | 3/4 | 2/4 |

**TABLE 3—Intracerebral Titration in Mice of Six Experimental Vaccines**

| Vaccine titrations | Mortality ratios of mice inoculated with 0.03 ml. of serial dilutions of vaccine | | | Residual $l.d._{50}$ of live rabies virus in each 0.03 ml. of vaccine |
|---|---|---|---|---|
| | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | |
| Rhozyme P-11 vaccine suspension | 3/5 | 0/5 | 0/5 | 15 |
| Rhozyme P-11 vaccine supernatant fluid | 2/5 | 0/5 | 0/5 | <10 |
| Protease 15 vaccine suspension | 1/5 | 0/5 | 0/5 | <10 |
| Protease 15 vaccine supernatant fluid | 1/5 | 0/5 | 0/5 | <10 |
| Heat inactivated vaccine suspension | 4/5 | 0/5 | 0/5 | 25 |
| Heat inactivated vaccine supernatant fluid | 4/5 | 0/5 | 0/5 | 25 |

mice which were inoculated intraperitoneally with six, 0.5 ml. doses of the control or experimental vaccines. The percentage of survivors in each group, subsequent to challenge with 500 $l.d._{50}$ of homologous virus, was calculated after 14 days of observation (table 4). When challenged, 80

**TABLE 4—Percentage of Survivors in Groups of Mice Vaccinated with Multiple Doses of Experimental and Control Vaccines and Challenged with Rabies Virus**

| Vaccines | Mortality ratios of mice challenged with 500 $l.d._{50}$ of rabies virus | Survivors 14 days after challenge (%) |
|---|---|---|
| Phenolized control vaccine suspension | 4/20 | 80 |
| Phenolized control vaccine supernatant fluid | 15/20 | 25 |
| Heat-inactivated control vaccine suspension | 15/20 | 25 |
| Heat-inactivated control vaccine supernatant fluid | 6/20 | 70 |
| Rhozyme P-11 vaccine suspension | 20/20 | 0 |
| Rhozyme P-11 vaccine supernatant fluid | 20/20 | 0 |
| Protease 15 vaccine suspension | 20/20 | 0 |
| Protease 15 vaccine supernatant fluid | 20/20 | 0 |

per cent of the mice vaccinated with a 10 per cent suspension of commercial phenolized vaccine of caprine origin and 70 per cent of those vaccinated with the supernatant fluid of a 10 per cent suspension of heat-inactivated vaccine survived. Both vaccines passed the screening potency test.[8]

When the mice vaccinated with a supernatant fluid of a 10 per cent suspension of heat-inactivated vaccine were challenged, 20 per cent of each groupsurvived. These vaccines did not pass the screening potency test.

Of the mice vaccinated with a 10 per cent suspension of supernatant fluid prepared from pooled cvs brain emulsion subjected to proteolysis with either Rhozyme P-11 or Protease 15, none survived when challenged. All four of the vaccines failed to pass the screening potency test.

The immunity established in those mice which were vaccinated with a supernatant fluid of a 10 per cent suspension of heat-inactivated vaccine indicates that the $l.d._{50}$ titer of the brain emulsion was adequate for the production of vaccine. From the titration of challenge virus, it was determined that the 1:79 dilution of brain emulsion used for the challenges contained 500 $l.d._{50}$ per 0.03 ml. dose.

## DISCUSSION

The titrations, in mice, of brain emulsions of five different strains of rabies virus which had been subjected to proteolysis, indicated that each of the five enzymes reduced the activity of each virus. The results obtained with each individual enzyme so closely approximated the others that a separate consideration of each enzyme is unnecessary. The effect of the enzymes on the viruses was not uniform insofar as each individual strain of virus was concerned. The percentage of decrease in the $l.d._{50}$ of active virus, following proteolysis at 37 C. for four hours, varied between strains from 88 to 99 per cent. This would indicate that each strain possessed a slightly different degree of resistance to the attenuating effects of the enzymes. It is obvious that elution of active virus was not successfully accomplished by proteolysis.

Infected rabbit brain emulsions of each virus, when subjected to a temperature of 37 C. for four hours in the absence of any enzyme, exhibited decreases in titer ranging from 37 to 80 per cent. An inverse relation was found to exist between the decrease in titer of a virus, attributable

solely to treatment at 37 C. for four hours, and the decrease attributed solely to the effect of the enzymes. When the decrease in titer of a virus by heat was great, the decrease by proteolysis was small but when the decrease in titer by heat was small, the decrease by proteolysis was greater. The action, described by previous investigators, of proteolytic enzymes on rabies virus is not due solely to the effect of the enzymes themselves, but also to the effect of the temperature at which all such proteolytic reactions take place.

The results of this investigation suggest that an increase in the elution of inactive virus was accomplished by proteolysis and that the quantity of inactive virus released by this method exceeds that which can be released solely by emulsification of infected brain tissue. Since an immune response might possibly be induced by inoculation of mice with suspensions of brain emulsion subjected to proteolysis, mice which survived the titrations of rabies virus brain emulsions subjected to proteolysis were challenged. Four $l.d._{50}$ of homologous virus was used as the challenge since it was believed that this concentration would provide a minimal lethal dose. A larger dose might possibly obscure any immunity that had been induced by a single intracerebral inoculation of 0.03 ml. of attenuated virus. A sufficient number of mice survived the challenge inoculation to indicate that some degree of immunity had been established. This immunity appeared to be associated with the amount of inactive virus known to be present in each of the original brain suspensions.

The results obtained in the challenge tests suggested that a higher level of immune response might have been induced if larger quantities or more concentrated suspensions of enzyme-brain emulsions had been used to inoculate the mice. Two experimental vaccines were prepared by proteolysis of brain tissue from the first rabbit passage of cvs virus. The incubation temperature for the proteolytic reaction in the production of the vaccines was 50 C. for four hours as compared to 37 C. for four hours in the elution of rabies virus by proteolysis. The incubation temperature was increased in order to inactivate the residual active virus which was present in the enzyme-brain emulsion preparations. It was assumed that the increased temperature increased the velocity of the reaction and did not increase the degree of proteolysis.[32]

When groups of mice were inoculated with a single 0.05 ml. dose of either Rhozyme P-11 or Protease 15 vaccine No. 1 and challenged 14 days later with serial $l.d._{50}$ dilutions of homologous virus, there was no immune response. A single dose of the commercial phenolized vaccine also produced unsatisfactory results.

In the attempts to immunize mice by multiple inoculations of the experimental vaccines, the results confirm the belief that Rhozyme P-11 and Protease 15 exerted an adverse effect on the antigenicity of the virus. When the mice which had been vaccinated with Rhozyme P-11 and Protease 15 vaccine preparations were challenged with a standard dose of 500 $l.d._{50}$ of homologous virus, none of them survived. This would indicate that the vaccines did not stimulate the production of neutralizing antibodies, or that the titer of the neutralizing antibodies that were produced was too low to withstand challenge. The latter deduction is probably more tenable, since a single intracerebral inoculation of similar enzyme-brain emulsion preparations induced an immune response sufficient to protect against 4 $l.d._{50}$. There is some indication, in the literature, that enzymes liberated from brain material used in the preparation of rabies vaccine might interfere with the antigenicity of the virus and prevent the stimulation of the production of neutralizing antibodies.[13] In the proteolysis of brain tissue, it would be logical to assume that foreign enzymes added to those liberated from the brain tissue could also exert a deleterious effect on the antigenicity of the virus.

With the commercial phenolized vaccine, the 10 per cent suspension was decidedly more antigenic than the supernatant fluid. The difference in the level of immunity induced by the vaccination of mice with these suspensions and supernatant fluids could possibly be explained as the effect of centrifugation. It has been reported that upon centrifugation of brain emulsions containing rabies virus, the brain tissue is deposited, taking with it over 90 per cent of the virus.[6]

With the heat-inactivated vaccines, the supernatant fluids were decidedly more antigenic than the suspensions. The amount of residual active virus present in the suspensions and the supernatant fluids

of the heat-inactivated vaccines was found to be approximately the same. A possible explanation might be that the virus, which was freed by the emulsion of the brain tissue and subsequently inactivated by heat, was of such particle size as to retard its sedimentation by centrifugation at low speeds. If such were true, a greater portion of freed inactive virus would remain in the supernatant fluid.

In the preparation of the enzyme vaccines and the heat-inactivated vaccines, the enzyme-brain emulsion and nonenzyme-brain emulsion preparations were incubated at 50 C. for one hour followed by 37 C. for three hours. Information available in the literature [28,33] indicates that one hour at 50 C. should have been sufficient to inactivate the virus. The results obtained in this study indicate that exposure of the cvs virus to 50 C. for one hour followed by 37 C. for three hours is insufficient to inactivate the virus.

It was found that the effect of the heat employed in the incubation of the enzyme and heat-inactivated vaccines accounted for over 99 per cent of the total amount of virus inactivated. This does not mean that the enzyme vaccines might not have contained a greater concentration of inactive virus. The method of calculating the amount of virus inactivated by the action of heat consisted of comparing the active virus titer of the vaccines to that of the original untreated brain emulsion. The original amount of active virus freed by proteolysis and subsequently inactivated by heat and enzymes, would not be detectable except by the demonstration of the increased antigenicity of such a preparation. This was not possible since the enzymes inhibited the ability of the virus to stimulate the formation of neutralizing antibodies. Investigation of the complement-fixing properties of the enzyme-brain emulsion preparations has been undertaken.

## Conclusions

1) Trypsin, Rhozyme P-11, Protease 15, Protease (trypsin), and Proteinase A exert an attenuating effect on the virulence of each strain of rabies virus tested.

2) The employment of the five proteolytic enzymes as a means of elution of active virus was not successful.

3) Intracerebral inoculation of mice with a 10 per cent suspension of rabies-infected rabbit brain subjected to proteolysis with either Rhozyme P-11 or Protease 15 may induce an immune response in the host sufficient to protect against a challenge of 4 $l.d._{50}$ of homologous virus.

4) Suspensions of brain material from rabbits infected with rabies virus subjected to proteolysis with either Rhozyme P-11 or Protease 15 are antigenically unsuitable for employment as vaccines.

5) Exposure of a 10 per cent suspension of rabies-infected rabbit brain emulsion to 37 C. for a period of four hours produces a marked decrease in the infectivity of the virus.

6) Exposure of a 10 per cent suspension of rabies-infected rabbit brain emulsion to 50 C. for four hours inactivates the virus.

7) Exposure of a 10 per cent suspension of rabbit brain emulsion of the cvs virus to 50 C. for one hour, followed by 37 C. for three hours, did not inactivate all of the virus.

## Summary

Rabbit brain emulsions of five strains of rabies virus were subjected to proteolysis with each of five enzymes. After completion of the reaction, serial dilutions of enzyme-brain emulsion preparations of each strain of virus were prepared.

Groups of mice were inoculated intracerebrally with each dilution and the survivors were challenged intracerebrally with 4 $l.d._{50}$ of homologous virus, 21 days subsequent to inoculation. Mice inoculated intracerebrally with enzyme-brain emulsion treated with either Rhozyme P-11 or Protease 15 developed an immunity.

Mice given a single intraperitoneal inoculation of a supernatant fluid of a 10 per cent suspension of rabies-infected rabbit brain emulsion subjected to proteolysis with either Rhozyme P-11 or Protease 15, showed no immunity upon challenge 14 days subsequent to inoculation. Multiple inoculations with the enzyme-brain emulsion preparations were equally fruitless.

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# Some Effects of a Bacterial Protease on the Complement-Fixing Antigenicity of Rabies Virus

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THE EFFECT of five proteolytic enzymes on the ability of rabies virus to induce the formation of neutralizing antibodies was reported in a preceding paper.[7] The original intention of this report was to study the effect of these enzymes on the complement-fixing antigenicity of the virus. However, all but one (Protease 15) were extremely anticomplementary and the study was limited to this bacterial protease.[5,6]

Specific complement-fixation test schedules have been developed for rabies,[1,3] but the schedule selected was the one described by Casals, *et al.*[2] in their specific complement-fixation test for infection with poliomyelitis virus. It is adapted to the effective utilization of small quantities of materials.

## MATERIALS

*Diluent.*—Physiological salt solution constituted the standard diluent for all serological procedures.

*Viruses.*—National Institutes of Health standard challenge virus No. 59, fixed, (CVS) and Lederle R174BK222AP99 (7-20-54) rabies street virus, (NY), were used as standard viruses. Each virus was identified as a rabies virus by means of the serum-virus neutralization test.[4,7]

*Antigens.*—The Protease 15 antigen was the same preparation as Protease 15 vaccine supernatant fluid No. 2 described in the preceding paper.[7]

Emulsions of brain tissue from rabbits infected with CVS and NY viruses were diluted to 10 per cent suspensions, centrifuged for 15 minutes at 1,200 × g, and the supernatant fluids decanted. These supernatant fluids were employed as active virus antigens. Similar preparations were made of noninfected rabbit brain tissue and employed as normal rabbit brain antigen.

The four antigens and their components were tested for anticomplementary and hemolytic activity[5] prior to employment in the complement-fixation tests.

*Sheep Erythrocytes.*—Fresh, citrated, normal sheep blood was used as the source of cells. The cells were washed three times in 4 volumes of diluent and suspended to a final concentration of 3 per cent, by volume.

*Hemolysin.*—Filtered (Seitz E-K), undiluted pooled serum of rabbits which had been sensitized to washed sheep erythrocytes was used as hemolysin. Titrations of hemolysin[5] were performed in which 1:2 dilutions of each antigen were substituted for the prescribed volume of diluent. One unit of hemolysin was defined as the smallest amount of hemolysin in 0.1 ml. capable of producing complete hemolysis of 0.1 ml. of a 3 per cent suspension of cells in the presence of an excess of complement and 0.2 ml. of a 1:2 dilution of antigen.

*Complement.*—Pooled normal serum, obtained from 8 guinea pigs, constituted the complement. Titrations of complement[2] (1:30 dilution) were performed in the presence of serial twofold dilutions, 1:2 through 1:16 of each antigen. One unit of complement was defined as the smallest amount of complement giving complete hemolysis after 18 hours at 4 C. in the presence of the antigen and 3 units of hemolysin.

*Serums.*—Lederle's concentrated anti-rabies serum (equine), dialyzed and diluted to 1:10, was used as equine anti-rabies serum. Pooled serum collected from 3 rabbits which had been immunized against rabies was employed as the rabbit anti-rabies serum. Equine serum, obtained from a horse with a normal clinical history, constituted the normal equine serum. Pooled serum collected from 3 normal rabbits was used as the normal rabbit serum. All serums were filtered (Seitz E-K) and positively identified as anti-rabies or normal serums prior to use in serological procedures, by testing for rabies-neutralizing antibodies using the CVS virus.[4]

## METHODS

"Box titrations"[2] were performed with twofold serial dilutions of Protease 15, and CVS- and NY-active virus antigens in the presence of twofold serial dilutions of equine and rabbit anti-rabies serum to determine the smallest quantity of each antigen which would fix complement in the highest dilution of each serum.

Complement-fixation tests were performed with Protease 15, NY- and CVS-active virus, and normal rabbit brain antigens. In one series, twofold serial dilutions, 1:5 through 1:640, of equine anti-rabies and normal serums were used. In a second series, similar dilutions of pooled rabbit anti-rabies and normal serums were employed. A 1:2 dilution of each antigen was used with the equine serums, and a 1:4 dilution with the rabbit serums. Normal rabbit brain antigen was not employed with the rabbit serums. Diluent and antibiotic solution controls were performed with each serum. Complement titrations were also conducted concurrently with the tests. Each series of tests was performed and read according to the methods of Casals *et al.*[2]

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**TABLE 1—Results of Complement-Fixation Tests Employing Enzyme and Active Virus Antigens**

| Serum | Titer of serum | | | | | |
|---|---|---|---|---|---|---|
| | Antigen dilutions | | | | Controls | |
| | Protease 15 | NY | CVS | Rabbit | Physiological salt | Antibiotic |
| | (1:2) | (1:2) | (1:2) | (1:2) | (1) | (1) |
| Equine immune | >640 | 0 | 0 | 0 | 0 | 0 |
| Normal equine | 5 | 40 | 40 | 20 | 20 | 20 |
| | (1:4) | (1:4) | (1:4) | (....) | (1) | (1) |
| Rabbit immune | >640 | 160 | 640 | .... | 0 | 0 |
| Normal rabbit | 5 | 80 | 80 | .... | 20 | 20 |

## RESULTS

On the basis of the results of the "box titration" of antigens, a 1:2 dilution of each antigen was used in the complement-fixation tests performed with equine serums. A 1:4 dilution of each antigen was determined to be suitable for use with the rabbit serums.

In the complement-fixation tests, the titer of each serum (table 1) was expressed as the greatest dilution of serum giving a 4 reading.[2] With the equine anti-rabies and normal serums, a titer of greater than 1:640 was demonstrated with the anti-rabies serum as compared with a titer of 1:5 with the normal serum with Protease 15 antigen, representing a greater than 128-fold difference in titer. With the equine anti-rabies serum, no titer was demonstrated with the other three antigens. With the pooled rabbit anti-rabies and normal serums, a titer of greater than 1:640 was demonstrated with the anti-rabies serum, as compared with a titer of 1:5 with the normal serum with Protease 15 antigen, representing a greater than 128-fold difference in titer. With the same serums, only a twofold difference in titer was obtained with the NY-active virus antigen, and an eightfold difference in titer with the CVS-active virus antigen.

None of the serums tested evidenced anticomplementary activity. The complement controls demonstrated that, in the absence of serum, a minimum of 1 unit of complement would have been available at the conclusion of the tests.

## DISCUSSION

The approximate concentration of active virus in the antigens had been previously determined.[7] The NY antigen contained about 390 times, and the CVS antigen about 3,900 times, more active virus than the Protease 15 antigen, per given quantity of antigen. The quantity of inactive virus in the Protease 15 and control antigens was not definitely known. However, proteolysis of brain tissue containing either CVS or NY virus with Protease 15 resulted in a greater than 99 per cent reduction in the amount of active virus in a given quantity of tissue, as compared with the titer of an identical quantity of untreated tissue.[7] Therefore, it is assumed that the Protease 15 antigen contained a greater concentration of inactive virus than the NY and CVS antigens. Based upon the results obtained in this study, it would appear that the active virus content of the antigens was not the determining factor in the comparative abilities of the antigens to fix complement, but that the amount of inactive virus in an antigen was related to its complement-fixing potential.

These assumptions would support the belief that an increase in the elution of inactive virus is accomplished by proteolysis and that the quantity of inactive virus released by this method exceeds that which can be released solely by the emulsification of infected brain tissue.[7] Although Protease 15 adversely affects the ability of the virus to induce the formation of neutralizing antibodies,[7] it appears that the enzyme does not interfere with its complement-fixing antigenicity.

## CONCLUSION

The enzyme, Protease 15, does not interfere with the complement-fixing antigenicity of the CVS strain of rabies virus.

## SUMMARY

A study was conducted to ascertain some of the effects exerted by proteolytic enzymes on the complement-fixing antigenicity of rabies virus. An antigen was prepared by the proteolysis of rabbit brain tissue infected with the National Institutes

of Health standard challenge virus No. 59, fixed (CVS) strain of virus, with a bacterial protease (Protease 15). The antigen was capable of fixing complement in the presence of equine and rabbit anti-rabies serum.

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# Neuropathology of Experimental Salmon Poisoning of Dogs

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SALMON POISONING was recognized many years ago as a distinct infectious canine disease enzootic along the Pacific coast from northern California to southern Washington.[23] However, the disease remained poorly defined pathologically until Cordy and Gorham [2] reported their observations in 1950. Subsequent studies at this laboratory [19] confirmed their account of the basic pathological pattern. These reports described lesions in the more ostensibly involved organs and tissues but did not include detailed consideration of the central nervous system (CNS). Only brief comment on the leptomeningeal reaction in 6 of 11 dogs with experimental salmon poisoning was reported.[19] This study of a large group of experimentally infected dogs was undertaken to establish the nature and distribution of lesions in the CNS.

## MATERIALS AND METHODS

Entire brains and portions of spinal cords from 62 Beagle dogs affected with experimentally induced salmon poisoning were used. Details of the methods used to infect the dogs and a description of the salient features of the experimental disease were reported.[18, 20, 21] The dogs were obtained from the breeding colony at this laboratory. Most of them were 4 to 9 months old at the time they were experimentally infected, although the group included animals whose ages ranged from 6 weeks to 4 years. Some infected dogs were allowed to die and others were killed, when moribund, by an intravenous injection of sodium pentobarbital. These animals were considered together for purposes of this study. The remaining dogs were killed at intervals after the characteristic febrile response occurred. A complete necropsy, including microscopic examination of many non-neural tissues, was performed on each dog. Gross and microscopic findings in each instance were characteristic of salmon poisoning uncomplicated by any recognized spontaneous disease.

Brains and portions of the spinal cords were fixed by immersion in buffered Orth's fluid or in neutral buffered 10 per cent formalin. The CNS of 1 infected pup was perfused with 10 per cent formol-saline solution to obtain good neuronal fixation. Usually, formalin-fixed tissue was hardened for three days in a 2.5 per cent aqueous solution of potassium dichromate. Blocks of tissue removed for routine preliminary examination included the cerebral cortex, basal ganglia, diencephalon, midbrain, cerebellum, and medulla oblongata. Brains were more generously sampled if lesions were found in sections prepared from these blocks. Complete coronal sections, through all major levels of the more severely affected brains, were used in studying the topographic distribution of the lesions. Blocks of spinal cord were removed from all major levels available, which, in many instances, consisted of upper cervical segments only. The tissue was embedded in paraffin, sectioned at 4 to 10 $\mu$, and stained with buffered azure-eosinate.[11] Certain sections were stained with Harris' hematoxylin and eosin and by a modification of Long's stain for reticulin.[17] The CNS of several uninfected Beagle dogs from the colony were used for control material.

## RESULTS

*Clinical Considerations.*—The general clinical signs and course of the fully developed experimental disease were characteristic of salmon poisoning.[2, 19, 23] One adult dog had convulsions terminally and several others were described as showing "paralysis" or weakness of the hind limbs for several days before death. The severely depressed attitude, characteristic of dogs with salmon poisoning, was a constant clinical feature.

*Gross Findings.*—At necropsy, brains from most dogs in advanced stages of the disease exhibited moderate and, occasionally, severe engorgement of meningeal blood vessels. In addition, appreciable engorgement of intracerebral vessels was observed in some dogs that died. Several brains appeared swollen and others were unusually "wet". The leptomeninges of 1 adult dog appeared opaque and somewhat granular. In others, the pia-arachnoid presented a slightly cloudy appearance. Detailed observations on this feature were not recorded. Coronal slices of the fixed brains revealed moderate distention

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of the lateral ventricles in five. Such dilatation was often asymmetrical, with medial displacement and flattening of the fornix on the side showing the greatest distention. The moderately dilated left lateral ventricle of 1 pup which died was almost completely filled with clotted blood. Portions removed from the spinal cord were free of lesions except for petechiae in the gray matter of the lumbar segments in 1 dog.

*Microscopic Findings.* — Microscopic lesions were found in 34 (91%) of 37 dogs that died. Changes were not recognized in 4 dogs killed during the first four days of disease. Of the 21 dogs killed after the fourth day, 15 (71%) had lesions (table 1). The neuropathological changes did not seem to be influenced by the duration of the incubation period or by the type of infectious material and route of administration used to produce the experimental disease. The following description of microscopic changes in the CNS is essentially a composite account based on findings in 49 dogs with lesions.

The principal lesions included: *(a)* diffuse and perivenous accumulation of mononuclear cells in the leptomeninges; *(b)* accumulations of mononuclear cells in sheaths of small and medium-sized intracerebral blood vessels; and *(c)* focal collections of glia or mesenchymal cells or both (so-called "glial nodules"). The frequency and intensity of these lesions varied considerably but their distribution was more or less constant. These microscopic changes were not seen in the control dogs.

*Leptomeninges.*—Accumulation of cells in the leptomeninges was a constant change and was the most conspicuous change observed (fig. 1). This change varied from scattered accumulations of activated histiocytes in the distended meshwork of the arachnoid to dense collections of mononuclear cells. In mild reactions, and presumably in the early stages of more severe leptomeningeal responses, histiocytes occupying the spaces of the arachnoid appeared to arise from the many hypertrophied cells of the arachnoid trabeculae. In brains showing more intense changes, numerous mononuclear cells were diffusely distributed throughout the pia-arachnoid, although the cells usually were concentrated perivenously. Such accumulations consisted of cells ranging from small lymphocytes to histiocytes and included many intermediate

Fig. 1—Cerebellar meninges of dog 59, which died on day 8, showing mononuclear cells in the distended meshes of the arachnoid and in the pia at the mouth of a sulcus. Azure-eosinate stain; x 55.

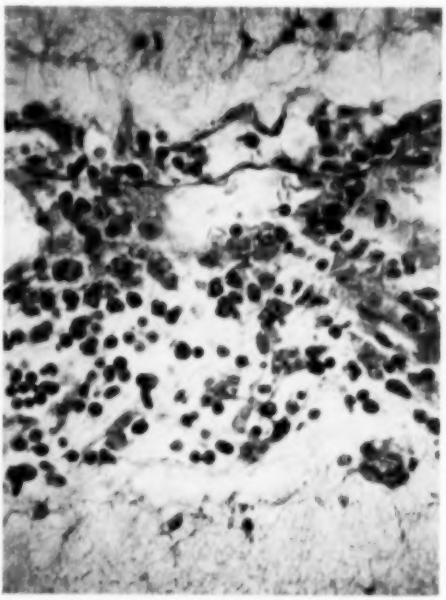


Fig. 2—Meninges in sulcus of frontal lobe of dog 43, which died on day 9. The infiltrate consists of histiocytes, large and small lymphocytes, and a few plasma cells. Azure-eosinate stain; x 375.

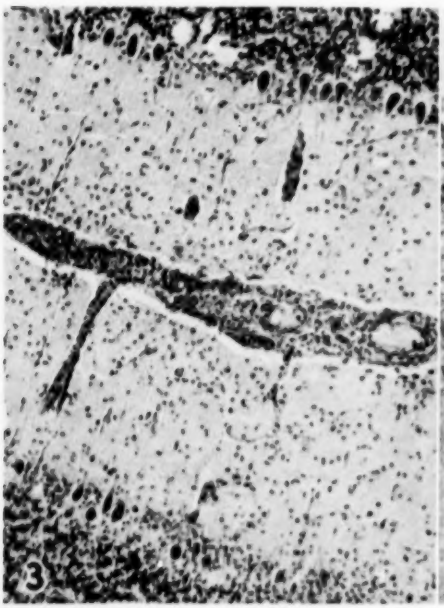
**TABLE 1—Relation of Duration of Disease to Neuropathological Changes in 62 Beagle Dogs with Experimental Salmon Poisoning**

| Number of dogs | Day of disease * | | | | | | | | | | | | | | | Totals | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | | |
| With lesions | | | | | | | | | | | | | | | | | |
| Died | | | | | | 2 | 1 | 9 | 9 | 6 | 3 | 4 | | | | 34 | 49 |
| Killed | | | | | 3 | 1 | 2 | 3 | 4 | | 1 | | | 1 | | 15 | |
| Without lesions | | | | | | | | | | | | | | | | | |
| Died | | | | | | | | 2 | | | | | | | 1 | 3 | 13 |
| Killed | 1 | | 1 | 2 | 1 | 3 | 1 | | | | | | 1 | | | 10 | |

* Day 1 of the disease is the first day of fever (39.6 C. or greater) after a variable incubation period.

forms of large lymphoid cells ("polyblasts" of Maximow) which possessed large, moderately chromatic, sometimes polymorphic, nuclei, and moderate-to-scanty amounts of blue to lavender cytoplasm (azure-eosinate stain) (fig. 2).

Plasma cells were conspicuous in several specimens and neutrophilic leukocytes occasionally were seen. Rarely, a few large binucleated cells were observed. Many of these cells in the leptomeninges doubtless were hematogenous. However, in numerous specimens where mononuclear cells were especially dense around veins, some cells appeared to arise from the vascular adventitia. Mitotic figures were frequent. Characteristically, in the denser perivenous accumulations especially rich in histiocytes and other large mononuclear elements, cell outlines were indistinct and the infiltrate assumed a somewhat "granulomatous" pattern (fig. 3). Diffuse, patchy infiltrations extended along the pia and frequently became coextensive with accumulations of similar cells in sheaths of veins penetrating the cortex. At focal points, inflammatory cells of the pia-arachnoid extended through the pia-glial barrier to the underlying cortex where they often became interspersed with prolifer-



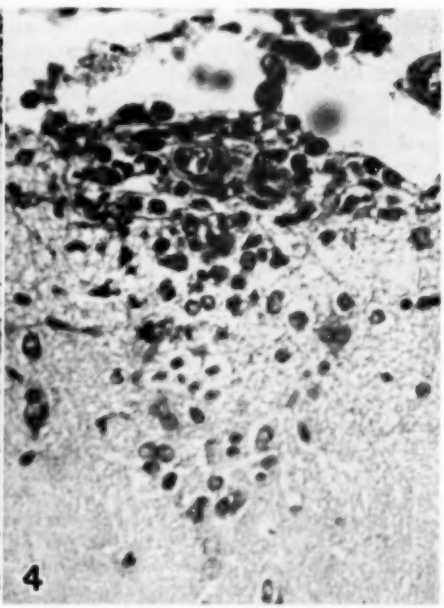




Fig. 3—Cerebellum of dog 112, which died on day 8. There is dense accumulation of mononuclear cells in meninges between folia with extension into the molecular layers. Azure-eosinate stain (Wratten filter 15); x 90.

Fig. 4—Cerebrum of dog 22, which died on day 10, showing invasion of temporal cortex by cells from the meninges. Azure-eosinate stain; x 425.

Fig. 5—Cerebellar meninges of dog 60, which died on day 8, showing three histiocytes which contain intracytoplasmic bodies resembling rickettsias (Neorickettsia helminthoeca). Azure-eosinate stain; x 1,500.

ated glia (fig. 4). This feature occurred with notable frequency in the molecular layer of the cerebellar cortex.

Scattered extravasated erythrocytes and, less often, focal hemorrhages occasionally were seen in the meningeal exudate. Hemorrhage and prominent erythrophagocytosis were observed in a brain in which a large blood clot was found in the left lateral ventricle. Small areas of necrosis in the inflammatory exudate were rare, but macrophages in the pia-arachnoid often contained engulfed nuclear debris. The small, often highly pleomorphic, intracytoplasmic bodies resembling rickettsias, now considered to be the etiological agent of salmon poisoning,[2,19] were seen in histiocytes and macrophages of the leptomeningeal infiltrate in 6 dogs (fig. 5). In one specimen, where the bodies were extremely numerous, the picture was reminiscent of the abundance usually seen only in lymphoid tissue in this disease.

Usually, the leptomeningeal response was uniformly distributed over all surfaces of the cerebral hemispheres. The reaction of the meninges overlying the brain stem generally was less intense, whereas the response was most intense over the cerebellum. Regardless of the topographic location, accumulations of mononuclear cells were more dense in the depths of fissures and sulci than over the surface of the brain. The cellular exudate was especially dense between cerebellar folia. In several instances, the meningeal reaction was limited to a few densely cuffed veins in such anatomic situations.

Leptomeningeal veins often were distended with erythrocytes and, in some specimens, their lumens contained numerous large lymphoid cells and foamy histiocytes resembling adjacent cells in the pia-arachnoid (fig. 6). Some cells displayed mitotic figures and others occasionally contained bodies resembling rickettsias. Intimal proliferation and vascular necrosis or thrombosis were not observed.

*Brain.*—In about one third of the brains, but predominantly in those of dogs that died, collections of mononuclear cells were seen in sheaths of scattered blood vessels varying in size from capillaries to medium-

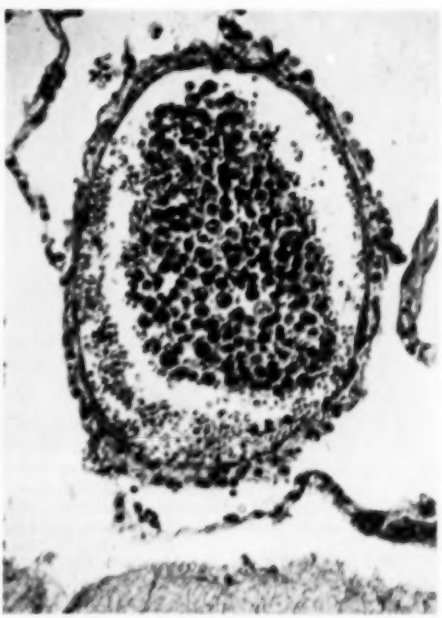


Fig. 6—Meningeal vein of frontal lobe with numerous histiocytes in the lumen from dog 38, which died on day 10. H & E stain; x 212.

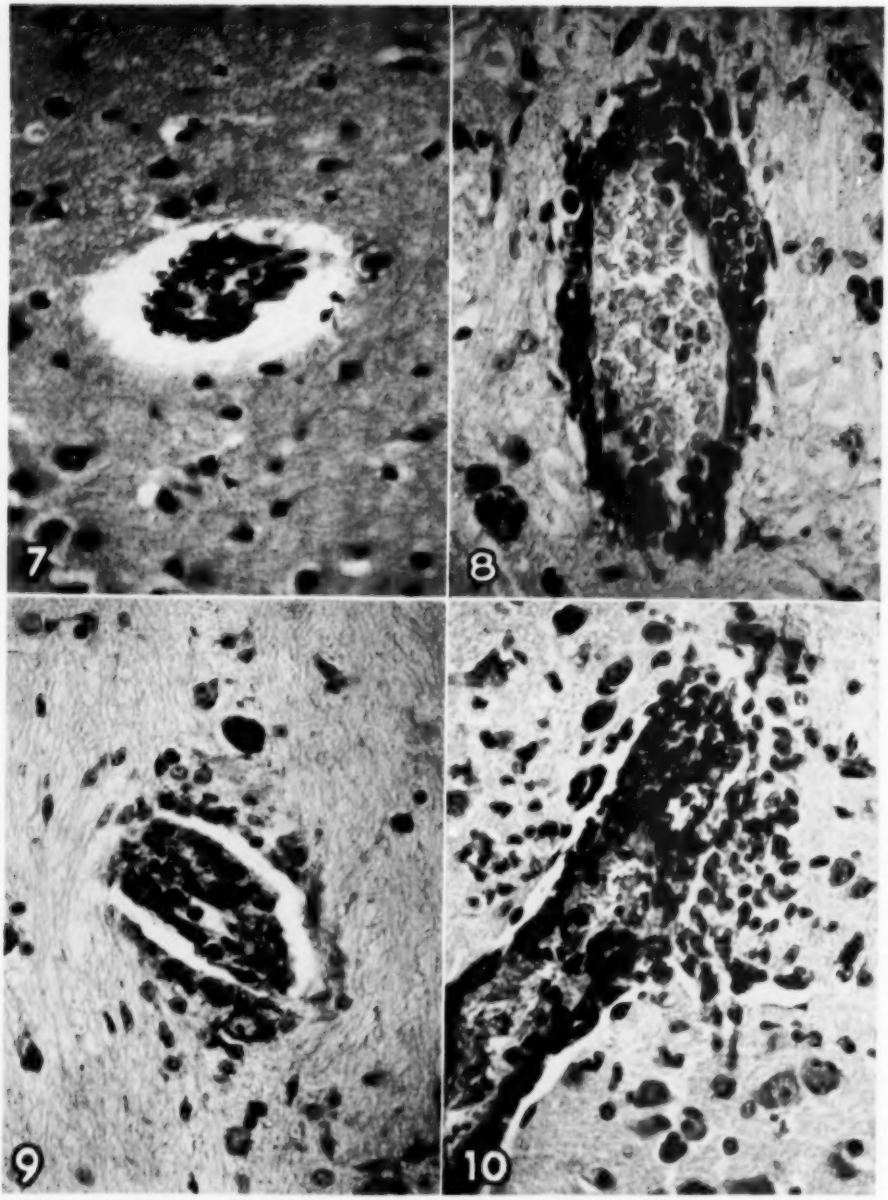



Fig. 7—Cerebrum of dog 59, showing proliferation of endothelial and adventitial cells of a small vessel of frontal cortex. H & E stain; x 390.

Fig. 8—Medulla oblongata of dog 60, showing dense arrangement of mononuclear cells in perivascular sheath. Some cells are probably hematogenous but may seem to be arising from the adventitia. Azure-eosinate stain; x 428.

Fig. 9—Pons from dog 24, which died on day 7, showing small vessel in pars basilaris pontis exhibiting prominent proliferation of adventitial cells which form a syncytium. Azure-eosinate stain (Wratten filter 15); x 425.

Fig. 10—Occipital cortex of dog 43, showing invasion of the periadventitial parenchyma by cells from the perivascular sheath. H & E stain; x 425.

sized veins (fig. 7). Small arteries were less often involved. Perivascular accumulations varied from a single row of cells around small vessels to three or four rows around larger vessels and were composed of tightly arranged, large mononuclear cells, histiocytes, and cells with elongated faintly chromatic nuclei (fig. 8). Small lymphocytes and plasma cells were less numerous. Cells displaying mitotic figures were common in some specimens.

On two occasions, bodies resembling rickettsias were demonstrated in perivascular histiocytes. Often, the distribution of cells assumed a focal or segmental pattern, resulting in prominent bulges in an otherwise unaffected sheath, as seen in transverse and tangential sections of the vessels. Accumulation of cells in vascular sheaths seemed to involve prominent swelling and proliferation of adventitial cells as well as exudation of hematogenous cells. Occasionally, when the perivascular reaction was intense, cell outlines were lost and the nuclei appeared in a syncytium of basophilic cytoplasm (fig. 9).

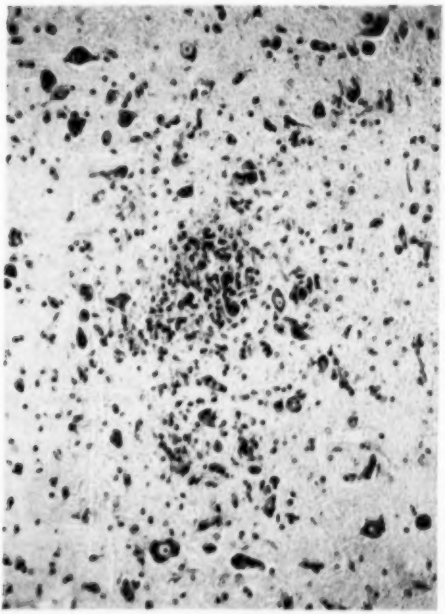


Fig. 11—Mononuclear cell nodule in tegmentum of pons of dog 25, killed on day 9. Origin from a vessel was apparent in a section prepared with Long's reticulum stain. Azure-eosinate stain; x 160.

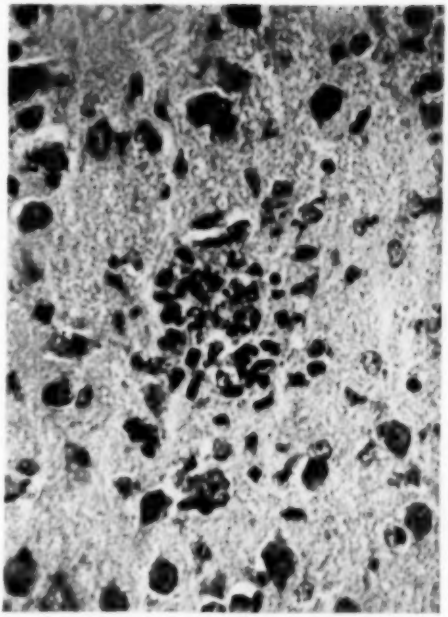


Fig. 12—Small nodule composed chiefly of mononuclear cells in occipital cortex of dog 43. H & E stain; x 425.

In sections prepared with the reticulum stain, mononuclear cells (except as mentioned later) were confined to reticular perivascular sheaths, where they occurred in the interstices of reticulin fibers as well as in clearly outlined, but not greatly distended, true perivascular spaces (Virchow-Robin spaces). Vascular endothelial swelling and proliferation were prominent only in capillaries and venules in the cerebral and cerebellar cortices. Concentric proliferation in small cortical vessels sometimes resulted in apparent occlusion by endothelial cells.

Venous and capillary congestion, sometimes accompanied by perivascular hemorrhage, was common. Hemorrhage was most frequent in the cerebral and cerebellar cortices and in the medulla oblongata, but was never extensive. In some specimens, large lymphoid cells and foamy histiocytes were abundant in lumens of parenchymal vessels as well as in meningeal veins. Vascular necrosis or thrombosis was never observed.

Generally, when vascular lesions were mild they were limited to small vessels of the cerebral cortex and were rarely found in the subcortical white matter. When

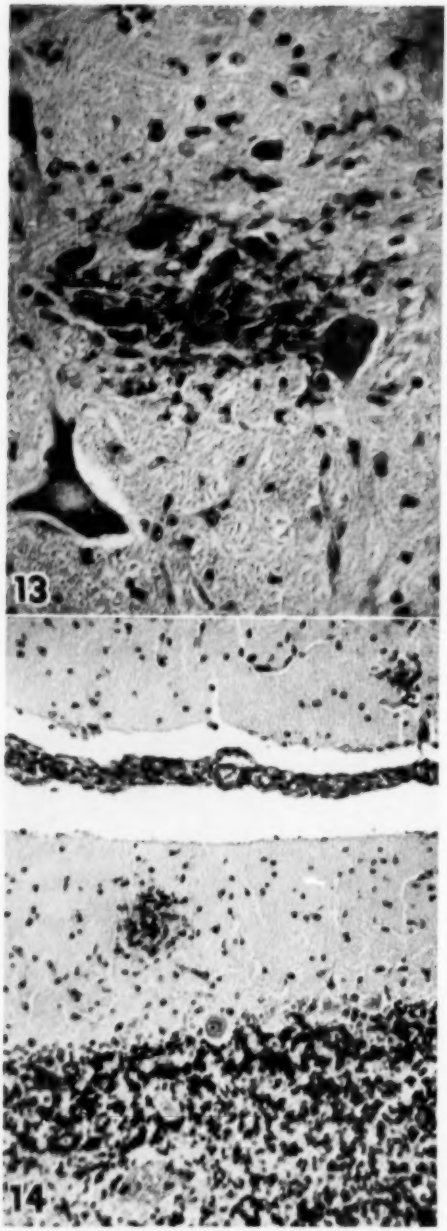


Fig. 13—Deeply basophilic syncytial nodule (granuloma) in region of dentate nucleus in cerebellum of dog 43. Azure-eosinate stain; x 340.

Fig. 14—Mononuclear cell nodules in the molecular layer of the cerebellum of dog 2, which was killed on day 9. H & E stain; x 180.

more intense, as in dogs that died, affected vessels were equally common in the diencephalon, midbrain, pons, medulla, and cerebellum. Vessels in the cerebellar white matter were involved as often as those in the cortex. Affected vessels were notably less frequent in the basal ganglia and hippocampus. These vascular alterations were unaccompanied by detectable changes in the surrounding parenchyma, other than scanty collections of activated microglia in some brains and questionable edema in others. However, cells in some vascular sheaths had penetrated the membrana limitans gliae and were spreading in the periadventitial brain substance either diffusely or in the form of nodules (fig. 10).

Sparsely distributed focal collections of glia or mesenchymal cells, or both, were present in more than half of the brains (fig. 11). Such foci were most prominent in dogs that died. However, in any given brain these nodular lesions were considerably less numerous than vascular changes. Many nodules were arranged either eccentrically or concentrically about small blood vessels. Such relationship to a vessel was not apparent in some nodules when the azure-eosinate stain was used. Nevertheless, with Long's stain, strands of reticulin suggestive of a vessel sheath sometimes were demonstrated within these nodules. Nodular lesions were either compact or loose and were usually composed of a few small pyknotic nuclei and numerous larger, round or oval, moderately chromatic nuclei with little or no discernible cytoplasm (fig. 12). Some larger nodules (85 to 120 $\mu$) possessed large, faintly chromatic, nuclei with poorly defined basophilic cytoplasmic processes that formed syncytial masses or granulomas (fig. 13). Although a few cells doubtless were glial, the majority appeared to be hematogenous, of adventitial origin, or from both sources. A less frequent type of nodule, usually smaller and less compact, was not related to blood vessels. These were composed chiefly of proliferated microglia, oligodendroglia, or both.

The extreme paucity of nodular lesions and their variable frequency in any given area of the brain made it difficult to determine a pattern of distribution. Nevertheless, a general idea was gained when all observations were considered. Nodular lesions, especially the larger type, were

most numerous in the midbrain, pons, medulla oblongata, and cerebellum. They were less frequently found in the diencephalon and cerebral cortex. An occasional nodule was seen in the hippocampus, but the basal ganglia were notably free of such lesions. Limited observations suggested that the inferior olivary nuclei tended to be more frequently and more intensely affected by nodular and diffuse inflammatory changes than other nuclear masses in the medulla. Elsewhere in the brain stem, similar sites of predilection were not detected. The molecular layer of the cerebellar cortex often displayed intense inflammatory changes in the form of diffuse infiltrations of mononuclear cells, proliferated glia and, sometimes, neutrophilic leukocytes, as well as in the form of nodules (fig. 14). To a lesser extent, nodules also were present in the cerebellar white matter and nuclei.

Mild-to-moderate regressive changes were recognized in neurons, but the precise extent of these alterations was difficult to assess in the material studied. Although an occasional nerve cell was surrounded by an inflammatory nodule, acute necrosis of neurons with or without neuronophagia was not seen. Swelling and proliferation of oligodenroglia were found in some brains, but marked changes in neuroglia were not recognized. Neurons, glia, vascular endothelium, and ependymal cells did not contain inclusion bodies.

Choroid plexuses in one third of the brains were sparsely to moderately infiltrated by mononuclear cells resembling those found in the leptomeninges (fig. 15). Occasionally, plasma cells were numerous and, in several specimens, neutrophilic leukocytes predominated. Often the infiltrating cells were arranged in an edematous stroma around widely dilated veins. Bodies resembling rickettsias were found in macrophages of the choroidal infiltrate in 5 animals. Epithelium of the plexuses rarely exhibited swelling and desquamation.

*Neurohypophysis.*—Complete sections of pituitary glands were available from 33 dogs. Lesions essentially identical with those in the brain were observed in the neurohypophyses of 11 dogs (fig. 16). Of these dogs, 9 died, and 2 were killed on the seventh and eighth days, respectively. Proliferation of the vascular adventitia was the predominant lesion, whereas mononu-

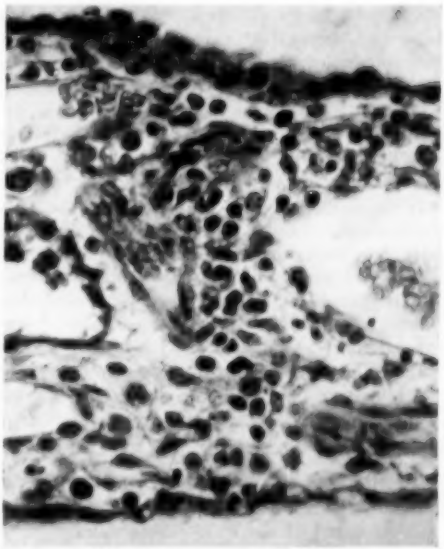


Fig. 15—Mononuclear cell infiltration around widely dilated vessels, and edema of the stroma, in choroid plexus of lateral ventricle of dog 72, which died on day 11. Azure-eosinate stain; x 425.

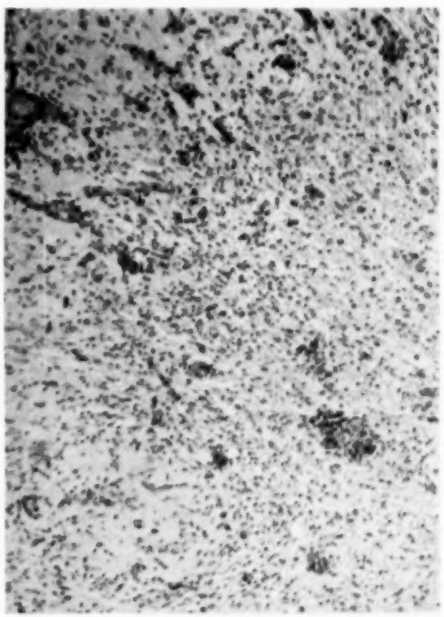


Fig. 16—Vascular adventitial cell proliferation and mild diffuse mononuclear cell infiltration of parenchyma of neurohypophysis of dog 99, which died on day 8. Azure-eosinate stain; x 85.

clear cell nodules were seen only occasionally. In half of the affected specimens, the parenchyma was sparsely infiltrated by plasma cells, lymphocytes, macrophages, and neutrophilic leukocytes. Bodies resembling rickettsias were demonstrated in macrophages in one specimen.

*Spinal Cord.*—The spinal cord was not involved except for minor changes in a few dogs. Leptomeningeal reaction was slight or absent and only an occasional vein exhibited perivascular collections of mononuclear cells. Engorgement of parenchymal blood vessels was variable and, in several dogs, was accompanied by perivascular hemorrhage. A few small, loose collections of glia occasionally were seen in the white matter.

## DISCUSSION

Although the intensity of the histopathological changes varied considerably, involvement of the CNS occurred with regularity (table 1). In general, the most intense inflammatory changes were seen in dogs that died after a typical clinical course. Lesions were proliferative in character with little evidence of degeneration and necrosis. This is consistent with the basic microscopic pattern of the disease as seen in other organs, although extensive necrosis may accompany the proliferative response in lymphoid tissue.[2, 8, 19]

The leptomeningeal reaction was an early and constant lesion. Its greatest intensity over the cerebellum was a distinctive feature of the neuropathological pattern. In early and mild responses, the microscopic picture was that of a reactive leptomeningitis with the formation of histiocytes from hypertrophied arachnoid cells in a manner similar to that described by Stone.[26] The numerous mononuclear cells that participated in the more intense inflammatory responses often were densely arranged, especially around veins, in a somewhat "granulomatous" pattern reminiscent of the focal lesions occasionally seen in other organs of these dogs.[8] Meningeal involvement appeared to be a primary rather than secondary reaction to intracerebral lesions, since the parenchyma underlying the meningitis often was free of changes. The nature of the cellular response and the morphological demonstration of bodies resembling rickettsias in inflammatory cells suggest that the meningeal reaction is specific (in the sense that it resulted from localization of the etiological agent in the meninges) and is not a general response to systemic disease.[5] Presumably, involvement of the leptomeninges, as well as other structures of the CNS, is incidental to the appearance of the etiological agent in the peripheral blood early in the course of the disease.[20, 24]

Compared with the meningeal lesion, intracerebral changes occurred less often and displayed greater variation in intensity. Some of the diverse morphological types of mononuclear cells in sheaths of blood vessels and infiltrating the parenchyma, either diffusely or as nodules, were considered to be hematogenous.[16] However, proliferation of adventitial cells often was the predominant vascular lesion, and appearances suggested that these cells also infiltrated the parenchyma. Since the majority of component cells of the proliferative nodular lesions appeared to be hematogenous, of adventitial origin, or from both sources, the designation, "glial nodule," so often applied to such cellular masses seemed inappropriate. The significance of the numerous large lymphoid cells and histiocytes in the lumens of meningeal and intracerebral blood vessels is not known. Such intravascular accumulations have not been observed in other organs.[8] This observation emphasizes the need for hematological studies which thus far have been neglected.

The neuropathological changes of salmon poisoning can be characterized morphologically as representing a mild-to-moderate nonsuppurative meningitis or meningoencephalitis. These CNS changes differ from those usually seen in naturally occurring viral infections of dogs, e.g. rabies,[10], canine distemper,[28] and infectious canine hepatitis.[22] Although the large cellular nodules suggest a similarity to lesions in the brain in toxoplasmosis,[7] the over-all neuropathological pattern of salmon poisoning is unlike that reported for cerebral toxoplasmosis in the dog.[6] In many respects, the inflammatory changes resemble those found in the CNS of man and in laboratory animals infected with the typhus-like rickettsias.[12–15, 27]

The etiological agent of salmon poisoning is presumed to be small intracytoplasmic coccobacillary bodies that characteristically occur in histiocytes and macrophages in lymphoid tissue of infected dogs.[2, 19] The name, *Neorickettsia helminthoeca*, was pro-

posed for this agent.[19,20] It is thought to be more closely related to a group of agents resembling rickettsias, which cause local or systemic disease in domestic animals,[1,4] than to the typical typhus-like rickettsias.[18] In contrast to infections with the latter organisms, little is known about the nature of the CNS lesions in systemic diseases associated with the organisms which resemble rickettsias. One of these agents, *Ehrlichia canis*, is considered to be the etiological agent of a canine disease which clinically is similar to salmon poisoning.[4,9] However, a detailed description of the lesions was not found which would allow anatomical comparison of the two diseases. Another of this group, *Cowdria ruminantium*, is the causative agent of heartwater, a septicemic disease of domestic ruminants in Africa.[4,9] Steck[25] found only minor neuropathological changes, although prominent clinical signs of CNS involvement characterize the disease. Pronounced leukostasis, as seen in other organs, with many small vessels often packed with macrophages, occurred in the absence of other lesions. In addition to leukostasis, Daubney[3] mentioned perivascular infiltration in the cerebral cortex, cellular exudation into the subarachnoid space, and satellitosis of neurons. Both authors observed bodies resembling rickettsias *(C. ruminantium)* in vascular endothelium, notably in the cerebral cortex and cerebellum. On the basis of these rather fragmentary observations, it is difficult to compare the neuropathological patterns of the so-called "animal rickettsioses" of Africa and salmon poisoning.

The generally mild nature of the CNS lesions is consistent with the absence of prominent clinical signs of neurological involvement in salmon poisoning. Nevertheless, in view of the pathological findings reported herein, critical evaluation of the clinical signs and physical findings for evidence of such involvement would seem to merit increased attention in future studies.

## SUMMARY

The brains and portions of the spinal cords from 62 Beagle dogs affected with experimental salmon poisoning were studied to determine the nature and distribution of lesions in the central nervous system. Engorgement of meningeal vessels was a common gross finding, especially in dogs that died. Microscopic changes were observed in 34 (91%) of 37 dogs that died, and in 15 (71%) of 21 dogs killed during advanced stages of the disease. These changes consisted of: *(a)* slight to moderate accumulation of mononuclear cells in the leptomeninges, which was most intense over the cerebellum; *(b)* cellular exudative and proliferative changes in sheaths of small and medium-sized intracerebral blood vessels; and *(c)* focal collections of glia, mesenchymal cells, or both (nodules). Intracerebral lesions were less frequent and less prominent than the leptomeningitis and were found most often in the cerebral cortex, brain stem, and cerebellum. Lesions similar to those in the brain were present in the neurohypophyses of 11 dogs. The spinal cord was not involved except for slight meningeal infiltration and an occasional vascular lesion in some animals.

These neuropathological changes differ from those reported for other natural infections of dogs but resemble lesions in the central nervous system of man and in laboratory animals infected with typhus-like rickettsias. It is difficult to determine, from available reports, whether there is any distinct resemblance to the neuropathological changes in heartwater or other so-called "animal rickettsioses." The generally mild nature of the lesions in dogs with salmon poisoning is consistent with the absence of prominent clinical signs of neurological involvement.

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# The Correlation Between Packed Cell Volume and Erythrocyte Number in Canine Blood

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THE DETERMINATION of the erythrocyte and leukocyte number is a time-consuming operation that requires considerable skill if reliable results are to be obtained. The limited time that practicing veterinarians can devote to hematological tests often prevents them from mastering the technique involved well enough to use the tests as valid diagnostic aids. The branch of veterinary medicine that benefits most from such tests is canine medicine.

Two procedures are essential to hematological study. One is the Wintrobe hematocrit, which provides valuable information regarding anemia, hemoconcentration, icterus, leukopenia, and leukocytosis. The other is the fresh blood film, which reveals the size, shape, and staining characteristics of the erythrocytes, the presence or absence in the peripheral blood of young forms of both the erythrocytic and the leukocytic series, and makes possible the differential leukocyte count.

These data can be gathered easily and rather quickly whereas the determination of the erythrocyte and leukocyte number is time-consuming. Tables that would tell the clinician the erythrocyte number for a given packed cell volume (PCV) would, therefore, be convenient.

Schalm and Wood [1] prepared such a table, in which the PCV obtained by the Wintrobe method can be used to estimate the erythrocyte number and hemoglobin concentration in canine blood. The data they used in preparing this table came from routine hematological tests made on 583 canine patients, of different breeds, sexes, ages, and health conditions, brought to the clinic of the School of Veterinary Medicine in Davis. The only evident inconvenience of this table is that the standard error of 0.74 million for the estimated erythrocyte count would allow a fluctuation in the number of erythrocytes greater than that permitted for technical error when actual counts are taken. In the case of low PCV readings, the possibility of such large variation can easily render the table useless.

The chance of error in determining the erythrocyte number is directly proportional to the number of the erythrocytes present in the counting chamber—that is, if the PCV is zero, the value of the erythrocytes is zero too. Thus, a table that would give the standard error for each estimated erythrocyte number obtained from a given PCV would be more reliable and useful.

## MATERIALS AND METHODS

Forty-four purebred, female Beagles between 1 and 2 years of age were used in this experiment. These dogs were kept on a nutritional regimen that included the feeding of a compound ration and one to two feedings weekly of freshly frozen beef heart, cheek, or oxtail at the rate of approximately 2 lb. weekly per dog. These dogs were relatively free from disease and parasites. Their care has been described by Andersen and Hart.[2] From these dogs, 562 PCV readings with corresponding erythrocyte counts were used for this study. Standard hematological techniques were employed as described by Wintrobe.[3] The hematocrit readings ranged from 26 to 62 mm.; 91.6 per cent of the readings were between 39 and 57 mm. The erythrocyte counts observed for certain PCV values (*i.e.*, PCV's of 35, 40, 45, 50, and 55) are shown in the scatterdiagram (fig. 1).

The data revealed that for any given PCV there was a considerable variation in the erythrocyte counts. This variation must, in the absence of anemic conditions, be ascribed to technical difficulties in determining the actual erythrocyte count. Clearly, the most accurate estimate of the erythrocyte value corresponding to a given PCV would be obtained by calculating the average of a large number of observed erythrocyte counts made for that PCV reading. The average for the counts obtained at each PCV given in figure 1 is indicated by a cross.

For reasons of brevity, let X represent a PCV reading and Y the corresponding erythrocyte count.

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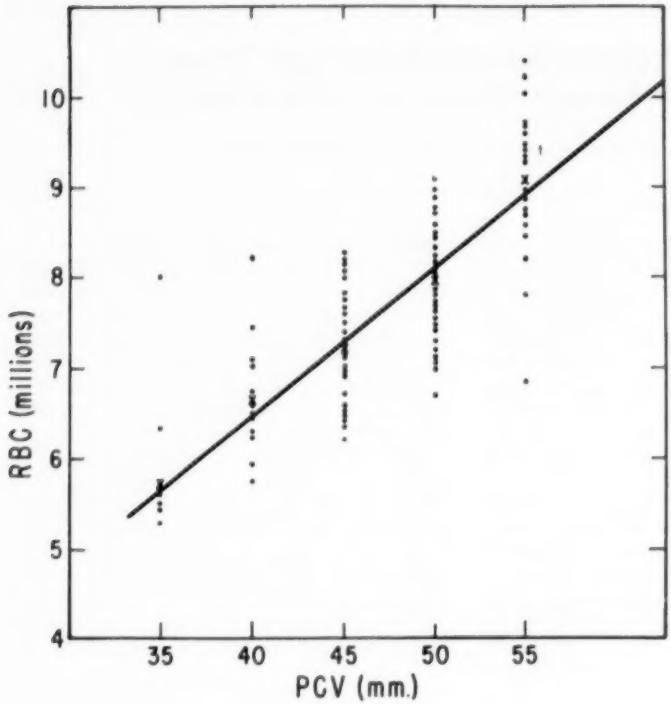


Fig. 1—Scatterdiagram of erythrocyte counts for certain PCV values.

**TABLE 1—Correlation Between PCV and Erythrocyte Number in Canine Blood***

| PCV X | Estimated erythrocyte No. $Y_e$ | Standard deviation $s(Y_e)$ | PCV X | Estimated erythrocyte No. $Y_e$ | Standard deviation $s(Y_e)$ |
|---|---|---|---|---|---|
| 10 | 1.62 | 0.0065 | 37 | 5.99 | 0.0239 |
| 11 | 1.78 | 0.0071 | 38 | 6.16 | 0.0245 |
| 12 | 1.94 | 0.0078 | 39 | 6.32 | 0.0252 |
| 13 | 2.11 | 0.0084 | 40 | 6.48 | 0.0258 |
| 14 | 2.27 | 0.0090 | 41 | 6.64 | 0.0265 |
| 15 | 2.43 | 0.0097 | 42 | 6.80 | 0.0271 |
| 16 | 2.59 | 0.0103 | 43 | 6.97 | 0.0278 |
| 17 | 2.75 | 0.0110 | 44 | 7.13 | 0.0284 |
| 18 | 2.92 | 0.0116 | 45 | 7.29 | 0.0291 |
| 19 | 3.08 | 0.0123 | 46 | 7.45 | 0.0297 |
| 20 | 3.24 | 0.0129 | 47 | 7.61 | 0.0304 |
| 21 | 3.40 | 0.0136 | 48 | 7.78 | 0.0310 |
| 22 | 3.56 | 0.0142 | 49 | 7.94 | 0.0317 |
| 23 | 3.73 | 0.0149 | 50 | 8.10 | 0.0323 |
| 24 | 3.89 | 0.0155 | 51 | 8.26 | 0.0329 |
| 25 | 4.05 | 0.0162 | 52 | 8.42 | 0.0336 |
| 26 | 4.21 | 0.0168 | 53 | 8.59 | 0.0342 |
| 27 | 4.37 | 0.0174 | 54 | 8.75 | 0.0349 |
| 28 | 4.54 | 0.0181 | 55 | 8.91 | 0.0355 |
| 29 | 4.70 | 0.0187 | 56 | 9.07 | 0.0362 |
| 30 | 4.86 | 0.0194 | 57 | 9.23 | 0.0368 |
| 31 | 5.02 | 0.0200 | 58 | 9.40 | 0.0375 |
| 32 | 5.18 | 0.0207 | 59 | 9.56 | 0.0381 |
| 33 | 5.35 | 0.0213 | 60 | 9.72 | 0.0388 |
| 34 | 5.51 | 0.0220 | 61 | 9.88 | 0.0394 |
| 35 | 5.67 | 0.0226 | 62 | 10.04 | 0.0401 |
| 36 | 5.83 | 0.0233 | | | |

* It should be carefully noted that the values of $Y_e$ given in this table predict the values which would have been obtained if the mean of a very large (or infinite) number of erythrocyte counts had been determined. Correspondingly, the $s(Y_e)$ values give the error applicable to these means. If, on the other hand, the mean of only a rather small number of erythrocyte counts for a given PCV is obtained, it would, of course, be subject to a much larger error.

From the 562 pairs of given X and Y values a line of regression

$$(1) \qquad Y_e = bX$$

can be obtained by the method of least squares (no constant term is included in (1), since we know that for PCV = 0 the value of erythrocytes = 0).

Calculating from the data the value of b, we find that

$$(2) \qquad b = \frac{\Sigma XY}{\Sigma X^2} = 0.162,$$

so that

$$(3) \qquad Y_e = 0.162\ X.$$

The value of $Y_e$ obtained from (3) for a given value of X represents an estimate of the actual (or mean) erythrocyte count for that PCV value. That this estimate is an excellent one is shown later. It is evident, however, from only a superficial inspection of the straight line shown in figure 1 that it is a good fit to the set of means indicated by the crosses.

Contrary to expectation, the variance of the observed Y values is approximately the same for all values of X. This means that the variance of erythrocyte counts obtained for a relatively low PCV reading is ordinarily not any less than that obtained for a high PCV reading. Thus, for example, the variance of ten erythrocyte counts for a PCV of 35 was found to be 0.81, while that of eight erythrocyte counts for a PCV of 57 was 0.64. Assuming then a common variance of the erythrocyte counts for all PCV readings, we find an estimate of that variance to be equal to

$$(4) \qquad s^2 = \frac{1}{n-2}\left[\Sigma Y^2 - \frac{(\Sigma XY)^2}{\Sigma X^2}\right] = 0.5400,$$

where n is the number of pairs of observations (in this case, n = 562).

To determine the accuracy with which formula (3) gives the desired averages, *i.e.*, actual erythrocyte counts, it is necessary to determine for each PCV reading the variance or standard deviation of the $Y_e$ values. It can be shown that for a given PCV value, say $X = X'$, this variance is given by

$$(5) \qquad s^2(Y_e) = \frac{X'^2 s^2}{\Sigma X^2},$$

where $s^2$ is the variance obtained from formula (4).

The values of $Y_e$, together with the corresponding standard deviations $s(Y_e)$, obtained from (5) are listed (table 1).

## Discussion and Conclusion

The presumptive values for the erythrocyte numbers obtained by Schalm and Wood were found to agree closely with the values presented in table 1 in spite of the different material used.

Thus, for example, we see from table 1 that, with a probability of 95 per cent, the mean erythrocyte number for a PCV of 35 must be between $5.67 - 2(0.0226)$ and $5.67 + 2(0.0226)$. Schalm and Wood give the corresponding presumptive erythrocyte number as 5.68, which is within the specified range. The rest of the values Schalm and Wood obtained are similarly within the range specified by table 1 of this paper with the exception of a few values corresponding to low PCV readings.

The following conclusions were reached:

1) The correlation existing between a PCV and the mean erythrocyte number in canine blood is fairly constant. While this has been demonstrated here for healthy dogs, it will ordinarily also apply to those suffering from disease since the size of the erythrocyte in dogs is not usually altered in case of disease. This statement agrees with the observation that anemia in dogs is usually of the normocytic normochromic type. In fact, the macrocytic type of anemia in dogs occurs rarely and the microcytic type of anemia due to chronic blood loss is seen mostly in the southern part of the United States where hookworms and other blood-sucking parasites are frequent.[4]

2) The standard error $s(Y_e)$ decreases with decreasing values of PCV because the accuracy of prediction of the erythrocyte number ($Y_e$) increases with smaller values of PCV (X), as we are certain that for X = 0 we must have Y = 0 and, consequently, $s(Y_e) = 0$.

3) The generally small standard error confirms the finding that table 1 allows a very precise prediction of the mean erythrocyte number from a single PCV reading, limiting the actual counting of erythrocytes to those rare cases in which clinical examination and observation of the stained blood film indicate it to be necessary.

## Summary

A statistical evaluation of 562 packed cell volume (PCV) readings of canine blood and corresponding erythrocyte counts has been presented.

The analysis proves that in the case of canine blood the correlation between PCV readings and the mean erythrocyte number is better than would appear from a superficial observation. The mean erythrocyte number can, therefore, be determined to a high degree of accuracy directly from a single reading of the PCV. The statistical procedures are described and a table shows the values obtained.

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[3] Wintrobe, M. M.: Clinical Hematology. 3d ed. Lea and Febiger, Philadelphia, 1951.

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# Chemotherapy of Canine Filariasis

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THE CHEMOTHERAPY of filaria infections of man or animals requires chemical agents which will destroy the adult worms. While the location of the adult worms varies markedly with the species, the microfilariae are usually found in the circulating blood. Much of the earlier work on this subject has utilized the microfilariae as the index of effectiveness of therapeutic agents. Unfortunately, this has not been a satisfactory method since the activity of chemicals against the adult filariae is not parallel to their activity against the microfilariae. The filariae are all phylogenetically closely related, which may be the reason many chemical agents show similar activity against the adults of several species. Moreover, there is much more similarity in the activity of many chemicals against adult filariae of several species than against the adults and microfilariae of any one species.

Since the filariae of man and of domesticated animals can not be made to develop in small laboratory animals, it becomes necessary, for chemotherapeutic studies, to utilize a species which occurs naturally in an animal which can be maintained in the laboratory. The most convenient species would be *Litomosoides carinii*, which parasitizes the cotton rat *(Sigmodon hispidus)*, but it is not found in Japan. In addition to those in wild animals, the nine species which occur in this country are: *Wuchereria bancrofti* and *Wuchereria malayi* in man; *Setaria digitata, Setaria marshalli, Setaria labiatopapillosa,* and *Onchocerca gutturosa* in cattle; *Setaria equina* and *Onchocerca cervicalis* in horses; and *Dirofilaria immitis* in dogs.

*Dirofilaria immitis*, which is common in the dog in Japan, was chosen from among these for therapeutic studies. The microfilariae of this species appear in the peripheral blood in the daytime as well as at night. The location of living adult worms is restricted to the right ventricle of the heart. Thus, the activity of a chemical against the microfilariae can be determined by making blood examinations before, during, and after treatment, and the fate of the adult filariae can be ascertained at necropsy of the animal at the end of treatment.

Of the large number of chemicals that have been used in the treatment of filariasis of man or domestic animals, only those few which have shown significant promise are enumerated. Among these compounds, some which have never been extensively used are: acridine dyes (against *D. immitis*[21, 30] and *W. bancrofti*[56, 60]); organic compounds of mercury (against *W. bancrofti*,[1, 58] *D. immitis*,[48] and *Litomosoides carinii*[48]); naphthaline (against *W. bancrofti*[29]); and formalin (against *S. equina*[55]).

The cyanine dyes are in this same category but deserve special mention. In 1947, Welch and his co-workers[86, 88] reported that 1' 1-di-B-ethoxyethyl-2, 2' carbocyanine chloride was conspicuously active against the microfilariae of *L. carinii* in the cotton rat. The next year, Peters[70] reported 1-ethyl-3, 6-dimethyl-2 phenyl-4-pyrimide-2-cyanine chloride as the most promising of this group. They have shown marked microfilaricidal activity against *W. bancrofti* in man and *D. immitis* in the dog. While they kill the adults of *L. carinii* in the cotton rat, they have essentially no effect on the adults of *D. immitis* in the dog, and nothing is known of their activity against adult filariae in man. They have not been studied further as they are too toxic for use.

## COMPOUNDS EFFECTIVE AGAINST FILARIA

Only three groups of compounds have been used extensively against filariasis of man or domestic animals: the piperazines and organic compounds of antimony (Sb) and of arsenic (As).

*Antimonials.*—Until recently, filariasis was treated almost exclusively with trivalent antimonials. Since Thiroux, in 1910, reported the destruction of the microfilariae of *W. bancrofti* in man, there have been many reports of the microfilaricidal action of antimony against: *W. bancrofti* —lithium antimony thiomalate (Anthiomaline[6-10]), antimoncolloid,[51, 52] sodium antimony tartrate,[69] ethylstibamine (Neostibosan);[16, 18, 29, 80] *Onchocerca volvulvus* —stibophen (Fuadin) and tartar emetic;[2] *D. immitis*—stibosol,[4] stibophen,[5, 82, 83, 88, 89] lithium antimony thiomalate,[10] sodium antimony tartrate,[32, 33] sodium antimony xylitol;[48] *L. carinii*—sodium antimony xylitol.[48]

The action of antimony against the adult worms has been less dramatic. However, the destruction or injury of the following adult filariae by antimonials has been

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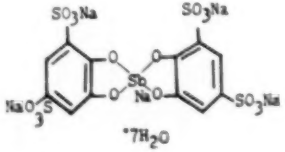
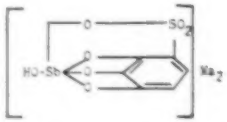
**TABLE 1—Effect on Microfilariae of Injections of Antimonial Preparations**

| Generic name and structure | Trade-name or common name | Sb (%) | Route of injection | Each dose (mg. Sb/kg.) | Total dose (mg. Sb/kg.) | No. of injections | No. of microfilariae: Pre-treatment | No. of microfilariae: Post-treatment | No. of days from treatment to disappearance of microfilariae | Dog (No.) |
|---|---|---|---|---|---|---|---|---|---|---|
| Sodium antimony-III *bis*-pyrocatechol-2-4-disulfonate (stibophen)  | Daviol | 1.0 | i.v. | 0.3–1.0 | 6.4 | 8 | 49 | 0 | 18 | 1 |
| | | | | 0.3–1.5 | 9.0 | 10 | 229 | 0 | 25 | 1 |
| | | | i.m. | 0.3–1.0 | 9.0 | 10 | 311 | 173 | .... | 1 |
| | | | | 0.3–1.5 | 8.0 | 8 | 37 | 0 | 19 | 1 |
| | | | subc. | 0.5 | 6.0 | 12 | 7 | 12 | .... | 1 |
| | | | | 0.75 | 9.0 | 12 | 8 | 0 | 25 | 1 |
| | | | | 1.0 | 8.0 | 8 | 153 | 0 | 18 | 1 |
| | | | | 1.5 | 4.5 | 3 | 81–543 | 0 | 5–6 | 3 |
| | | | | 1.5 | 6.0 | 4 | 1–272 | 0 | 6–8 | 5 |
| | | | | 1.5 | 7.5 | 5 | 55–199 | 0 | 9–12 | 3 |
| | | | | 1.5 | 9.0 | 6 | 357 | .... | 11 | 1 |
| | | | | 1.5 + 50 mg. SMT* /kg. | 12 + 400 mg. SMT/kg. | 8 | 49 | 0 | 18 | 1 |
| | | | | 1.5 + 50 mg. SMT/kg. | 12 + 400 mg. SMT/kg. | 8 | 27 | 1 | .... | 1 |
| | Fuadin | 0.85 | subc. | 1.5 | 13.5 | 9 | 26 | 0 | 17 | 1 |
| | | | | 1.5 | 12.0 | 8 | 59–82 | 0 | 8 | 2 |
| | | | i.v. | 1.5 | 43.5 | 29 | 13 | 0 | 59 | 1 |
| HO · CH · COONa<br>HO · CH · COOSbO · ½ $H_2O$ | Sodium antimony tartrate | 38.3 | i.v. | 1.0 | 10.0 | 10 | 52 | 48 | .... | 1 |
| | | | | 1.5 | 4.5 | 3 | 566 | 0 | 4 | 1 |
| | | | | 1.5 | 6.5 | 4 | 10–161 | 0 | 5–7 | 2 |
| | | | | 1.5 | 9.0 | 6 | 6 | 0 | 7 | 1 |
| Colloidal antimony powder + glucose | Antimon-colloid | | i.v. | 1.5 | 4.5 | 3 | 213 | 50 | .... | 1 |
| | | | | 1.5 | 6.0 | 4 | 12–552 | 0 | 7–9 | 2 |
| | | | | 1.5 | 6.0 | 4 | 12 | 0 | 5 | 1 |
| | | | | 1.5 | 9.0 | 6 | 62 | 0 | 8 | 1 |
| | | | | 3.0 | 6.0 | 2 | 71 | 0 | 4 | 1 |
| | | | | 4.5 | 4.5 | 1 | 45 | 0 | 3 | 1 |
| Sodium antimonyl pyrogallol sulfonate  | Lyoantimon | 1.0 | subc. | 1.5 | 9.0 | 6 | 1–12 | 0 | 6 | 2 |
| | | | | 1.5 | 13.5 | 9 | 62 | 0 | 10 | 1 |
| | | | | 1.5 | 22.5 | 15 | 1 | 0 | 17 | 1 |
| | | | | 3.0 | 9.0 | 3 | 1–121 | 0 | 3 | 4 |
| | | | | 3.0 | 12.0 | 4 | 68–139 | 0 | 4 | 2 |
| | | | | 5.0 | 5.0 | 1 | 283 | 167 | .... | 1 |
| | | | | 5.0 | 10.0 | 2 | 455 | 0 | 2 | 1 |
| | | | | 5.0 | 15.0 | 3 | 3–403 | 0 | 3 | 5 |
| | | | i.v. | 5.0 | 15.0 | 3 | 188 | 2 | .... | 1 |
| | | | subc. | 10.0 | 10.0 | 1 | 249 | 9 | .... | 1 |
| | | | | 15.0 | 15.0 | 1 | 593 | 0 | 3 | 1 |

* SMT = sulfamethathiazole

reported: *O. volvulus*—stibophen and tartar emetic;[2] *D. immitis*—antimoncolloid [77,78] and tartar emetic;[2] and *L. carinii*—stibamine (Neostam) and ethylstibamine.[15]

Of particular significance have been the recurrent reports of the sterilization of adult females, without their destruction, by organic compounds of antimony.

*Piperazines.*—Hewitt and his co-workers, in 1947, found the microfilaricidal action of the substituted piperazines and reported that 1-diethylcarbamyl-4-methylpiperazine hydrochloride (diethylcarbamazine, Hetrazan, Caricide) was the most active. Its microfilaricidal action against many species is now recognized; *i.e.*, against *L. carinii*,[25,26,28] *D. immitis*,[26,28] *W. bancrofti*,[60,72] *W. malayi*,[87] *Loa loa*,[77] and *O. volvulus*.[11] But, as pointed out by the discoverers of this drug,[26,28] the action against the adult worms is at best uncertain. In Japan, the 1-diethylcarbamyl-4-methylpiperazine citrate (Supatonin) is the preferred drug against *W. bancrofti* [22,34,37,38,75] and *W. malayi*.[22,73,74] The author [39] has shown that, like diethylcarbamazine and the trivalent antimonials, it is primarily microfilaricidal.

*Arsenicals.*—Apparently Ikegami,[31] in 1920, was the first to use arsphenamine (Arsaminol) against *W. bancrofti* in man. Subsequently, many arsenicals have been used.[12,13,53,57,70,71,79,84] Arsphenamine,[24] Atoxyl,[25] and neoarsphenamine [25] have also been used in canine filariasis. Although clinical improvement has been reported by many, only in a few cases was any diminution or elimination of the microfilariae reported [31,57,70,79] and even then they diminished slowly, in sharp contrast to the results following the use of trivalent antimony. There was no direct evidence that the adult filariae had been destroyed and little consideration was given to this possibility. Rather, the clinical improvement was largely assumed to be due to the tonic effect of the arsenic.[24,25] Accordingly, the arsenicals were not seriously considered as specific therapeutic agents prior to 1945.

In 1945, Otto and Maren [61] called attention to the lethal effects of the phenyl arsenoxides on the adult filaria and introduced p-[bis-(carboxymethylmercapto)-arsino]-benzamid (thiacetarsamide, arsenamide, Caparsolate sodium). They found little evidence of direct microfilaricidal activity against either *L. carinii* in the cotton rat or *D. immitis* in the dog. Rather, the microfilariae appear to have died off slowly over a period of four months or more after the destruction of the adults by this arsenical.[64] However, the microfilariae of *W. bancrofti* disappeared gradually during the 15-day course of treatment.[64,66,67,81]

The author, in the course of the present studies, has determined the extent of the microfilaricidal action of a great many chemicals which have been used previously and has studied the effect of some of them on the adult worms. One arsenical, dichlorophenarsine hydrochloride (Filarsen), appears to kill the adult worms regularly with as little as three daily intravenous doses and to be reasonably well tolerated by the host.

## MATERIALS AND METHODS

Dogs infected, both naturally and experimentally, with *D. immitis* were used. All drugs were given daily by injection. Drugs which had been used previously were tested here in the previously reported dosage schedules as well as at several times those doses.

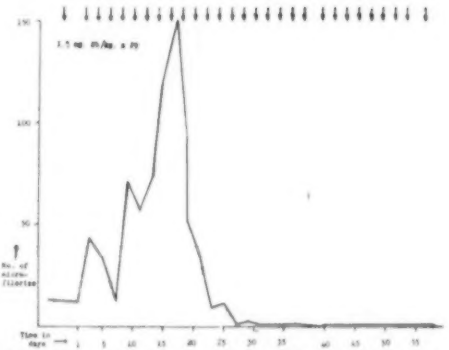
Microfilaria counts, from thick blood smears containing 0.01 cc. of blood, were made before treatment and daily during the course of treatment. To avoid any possible distortion due to periodicity, all blood smears from any given dog were made at the same time of the day. The animals were electrocuted at varying periods after treatment, at which time the right ventricle of the heart and the pulmonary artery were examined for adult worms. The worms recovered were placed in warm physiological saline solution in order that any sign of movement might be detected.

In view of the report by Brown and Sheldon [5] that the activity of antimony was enhanced by the concurrent use of sulfonamides, experiments were set up to compare the antimony blood levels in dogs receiving stibophen intramuscularly with and without concurrent intravenous injection of sulfamethylthiazol. The antimony determinations were made by Maren's colorimetric method on the rhodamine B complex with antimony.[50]

## RESULTS

*Microfilaricidal Activity.*—In most instances, the microfilariae completely disappeared shortly after the completion of treatment with the antimonial compounds. The rate of disappearance appears to follow three basic patterns regardless of whether the drug is given intramuscularly, subcutaneously, or intravenously (table 1). With the lower doses, there may be an initial increase in the number of microfilariae in the circulating blood, followed by a fluctuating number over a period of a week or two, followed, finally, by their dis-

appearance or reduction to a very low number. With a somewhat larger dose, there may be a slight initial increase followed by a rapid reduction and disappearance. With an initially heavy dose or doses (5.0 to 10.0 mg. of Sb/kg.), the microfilariae diminish rapidly and disappear without any initial increase. In some instances, when the early treatment has finally produced a gradual reduction in microfilariae, those remaining appear to be somewhat resistant to the antimony so that even with continuing treatment they are not eliminated (graph 1). Furthermore, the effect of the antimony on the microfilariae continues for only a short time. When a single dose or a few doses are given in rapid succession, the microfilariae begin to increase in numbers after the third or fourth day of treatment.



Graph 1—Persistence of microfilariae (dog 11) despite the continued administration of antimony (stibophen). Arrows indicate the times of treatment.

Although a previous report [5] has indicated that sulfonamides may enhance the microfilaricidal activity of trivalent antimony, no such evidence was obtained in these experiments (table 1). The administration of stibophen in daily doses (1.5 mg. of Sb/kg.) eliminated the microfilariae, but the simultaneous administration of sulfamethylthiazole (50 mg./kg.) did not increase the rate of microfilaria destruction and may even have retarded it. Accordingly, antimony blood levels were obtained on 4 dogs given stibophen (1.5 mg. of Sb/kg.) and then, 15 days later, the same dosage of stibophen plus 100 mg./kg. of sulfamethylthiazole. The results (table 2) suggest that the sulfonamide depressed the antimony blood content. This appears to be true for both the plasma and the cellular elements.

Of the nonmetallic compounds, acriflavine, naphthalene, cyanin dyes, and formaline had relatively little effect upon the microfilariae in the doses given (table 3). The slight reduction in the number of microfilariae following treatment with acriflavine and naphthalene, in a few of the dogs, was not comparable to that following the use of trivalent antimonials. The effect of 1-diethylcarbamyl-4-methylpiperazine citrate was less than has been reported. Nevertheless, there was a consistent reduction in the number of microfilariae (table 4). The reduction was marked when doses of 100 to 200 mg./kg. were given daily, but occurred also with daily doses of 50 mg./kg. (table 4).

The arsenicals had comparatively little effect upon the microfilariae (table 3). The greatest microfilaria reduction was in the dog receiving Carbamisin, although there appeared to be some reduction effected by neoarsphenamine, Solminol, and Neostibarsen. The latter is an organic compound of both antimony and arsenic.

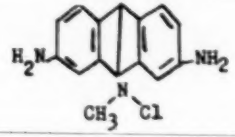
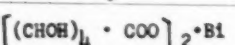
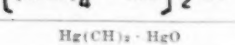
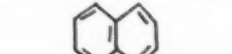
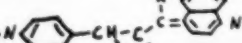
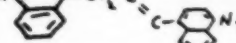
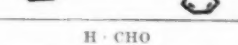
There is a sharp contrast between the failure of dichlorophenarsine to reduce the number of microfilariae and their marked reduction, after an initial increase, by Neostibarsen.

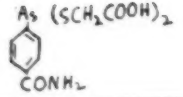
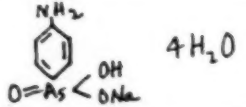
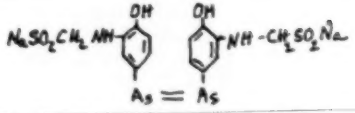
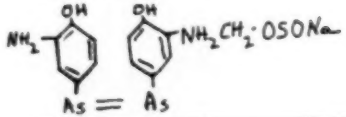
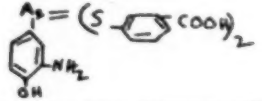
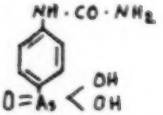
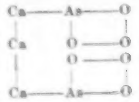
*Activity Against Adult Filariae.*—In the naturally infected dogs treated with either of two antimonials and necropsied shortly after completion of treatment (table 5, dogs 2–8; table 6), dead worms were recovered from only 1 (table 5, dog 7).

TABLE 2—Concentration of Antimony in Blood (μg./5.0 cc.) After an Injection of 1.5 mg. of Sb/kg. only, and After the Same Dose of Sb in Combination with 100 mg./kg. of Sulfamethylthiazole (SMT)

| Dog (No.) | | Time (hours) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.25 | 0.5 | 1.0 | 2.0 | 3.0 | 6.0 | 12.0 | 24.0 | 36.0 |
| 1 | Sb only | 11.40 | 8.30 | 7.80 | 6.40 | 3.80 | 3.00 | 1.75 | 3.50 | 1.10 |
| | with SMT | 8.70 | 7.40 | 6.30 | 4.50 | 3.70 | 2.00 | 1.20 | 1.44 | 1.27 |
| 2 | Sb only | 7.10 | 6.85 | 4.50 | 4.26 | 3.90 | 1.63 | 1.47 | 0.78 | 1.86 |
| | with SMT | 5.50 | 5.00 | 4.65 | 2.41 | 1.80 | 1.48 | 1.46 | 0.82 | 1.14 |
| 3 | Sb only | 18.20 | 10.20 | 8.10 | 5.25 | 2.23 | 1.80 | 1.00 | 2.05 | 6.10 |
| | with SMT | 8.30 | 6.70 | 5.55 | 4.17 | 2.10 | 0.85 | 1.00 | 1.40 | 1.77 |
| 4 | Sb only | 10.00 | 9.60 | 7.90 | 6.75 | 4.90 | 3.18 | 2.30 | 1.60 | 0.44 |
| | with SMT | 9.60 | 6.10 | 5.85 | 4.75 | 3.47 | 1.96 | 1.083 | 2.16 | 1.33 |

TABLE 3—Effect of Treatment of Microfilariae in Dogs, with Several Compounds Other than Antimonials

| Type | Name | Structure | Dose range per kg. × No. of injections | No. microfilariae (min.–max.) Pre-treatment | Post-treatment | Dog (No.) |
|---|---|---|---|---|---|---|
| Acridin dye | Acriflavine (Trypaflavin, 0.5%) |  | 0.3 cc. × 14–1.0 × 1 | 98–301 | 69–400 | 5 |
| | Pansecal | | 0.6 cc. × 10–2.0 × 15 | 11–458 | 20–71 | 5 |
| Bismuth | Syphrose |  | 4 mg. Bi × 10–0.3 × 10 | 6–107 | 9–235 | 5 |
| Mercury | Luestin |  | 4 mg. Hg × 10–1.0 × 10 | 35–70 | 27–130 | 5 |
| Naphthalene | Naphthalin olive (10%) | | 0.8 cc. × 15–2.3 × 15 | 3–248 | 2–81 | 6 |
| Cyanin dye | Lumin |  | 30 mg. × 14–20 × 14 | 10–156 | 9–316 | 5 |
| Formalin | Formalin (2%) | | 1 cc. × 10–2.3 × 10 | 9–358 | 6–320 | 5 |
| Arsenic | Dichlorophenarsine (Filarsen) |  | 1 mg. As × 3 | 5–488 | 5–493 | 16 |
| | Oxophenarsine (Mapharsol) |  | 1 mg. As × 3 | 29 | 38–48 | 2 |
| | Luargol (Neostibarsen) |  | 1.0 mg. As<br>1.3 mg. Sb } × 3 | 23 | 5 | 1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Arsenic | Thiacetarsamide (Caparsolate sodium) |  | 1 mg. As × 3 | 1 | 2 | 1 |
| | Sodium arsanilate (Atoxyl) |  | 1 mg. As × 3 | 30 | 26 | 1 |
| | Sulfarsphenamine (Myochramonol) |  | 1 mg. As × 3 | 12 | 13 | 1 |
| | Neoarsphenamine (Neoarsamine, Neoarsamanol) |  | 7 mg. As × 3 | 70 | 40 | 1 |
| | Solminol |  | 2 mg. As × 3 | 280 | 141 | 1 |
| | Carbamisin |  | 12 mg. As × 15 | 102 | 7 | 1 |
| | Mamol |  | 10 mg. As × 3 | 15 | 25 | 1 |

Even from this dog, only 4 of the 27 worms recovered were dead. However, only 3 living worms were recovered from the animals which were allowed to live four months longer (table 5, dogs 1 and 9–13). A considerable number of dead worms were so fragmented that no count could be made. Young worms, just before maturity, are more difficult to kill than fully mature worms; therefore, a more rigorous evaluation of the anthelmintic activity is afforded by using such worms for testing. Accordingly, 3 dogs which had been experimentally infected were treated with Lyo-antimon while the worms were believed to be still immature. This belief was confirmed at necropsy. One animal, killed three days after the completion of treatment, yielded 54 living, immature worms (table 7, dog 1), while 2 animals, killed over four months later, yielded no living worms and many fragments of dead worms. It is evident that antimony is capable of killing both mature and immature worms in the heart if sufficient time is allowed. However, all the dogs (tables 5–7) suffered toxic effects from the antimony, and it is doubtful if such doses could be used in routine practice.

The signs of intoxication were vomiting, loss of appetite and vitality, fever, arthritis, discharge from the eyes, and orchitis in male dogs. All dogs which were given more than three or four injections (table 5) of Antimoncolloid (1.5 mg. of Sb/kg.) or Lyo-antimon (5.0 mg. of Sb/kg.) became so weak (tables 6 and 7) that it would have been impossible to continue the treatment except in experimental dogs. There was a marked decrease of the total leukocyte count with some decrease of erythrocytes, platelets, hemoglobin, and serum protein; the sedimentation rate was increased, probably because of the decrease of fibrinogen. Damaged neutrophils (principally with basophilic granules) and anisocytosis, poikilocytosis, and punctate basophilia of erythrocytes were also observed.

Only living worms were recovered (table 4) at necropsy of 3 of the dogs receiving the piperazine 31, 36, and 103 days, respectively, after completion of treatment.

Among the series of treatments with arsenicals (table 8), only three were followed by the recovery of dead worms. Necropsy, ten days after three doses of oxyphenarsine hydrochloride, revealed 14 living and 6 dead worms (table 8, dog 1); a similar procedure with Neostibarsen yielded 9 living worms and 1 dead one (table 8, dog 2). After the simultaneous use of dichlorophenarsine hydrochloride and stibophen, only dead worms were recovered (table 8, dog 12).

Further studies indicated that the worms were killed by the dichlorophenarsine. Dogs which received dichlorophenarsine in three or more daily doses of 1.0 mg. of As/kg. and were necropsied ten days after the start of treatment yielded only dead worms. The 1 dog (8) that received three daily doses of 1.0 mg. of As/kg. and was killed the fifth day after the first injection yielded only living worms. Even the 3 dogs (10, 11, 12) which received only two daily doses of 1.0 mg. of As/kg., and were killed 20 to 30 days after the first injection, produced only dead worms, while a fourth (dog 13), killed 15 days after the first injection, yielded 3 dead and 2 living worms. A single dose of 3.0 mg. As/kg. destroyed all the worms in ten days (dog 7). However, in the dog which died only two days after this dose, 1 dead and 4 living worms were found (dog 9). The only animal examined less than ten days after the start of treatment, which yielded only dead worms, was one (dog 3) which received two daily doses of 2.0 mg. As/kg. It would appear that this arsenical can kill the adult worms quickly if the dose is

**TABLE 4—Effect on Microfilariae and Adult Worms of Daily Oral Doses of 1-Diethylcarbamyl-4-Methylpiperazine Citrate (Supatonin)**

| Dog (No.) | Daily dose (mg./kg.) × No. of treatments | No. of microfilariae | | No. of days from treatment to necropsy | Adult worms | |
|---|---|---|---|---|---|---|
| | | Pre-treatment | Post-treatment | | Alive | Dead |
| 1 | 200 x 5 | 67 | 1 | 31 | 7 | .... |
| 2 | 100 x 5 | 102 | 34 | .... | .... | .... |
| 3 | 50 x 5 | 18 | 3 | .... | .... | .... |
| 4 | 50 x 5 | 55 | 31 | .... | .... | .... |
| 5 | 30 x 5 | 52 | 14 | .... | .... | .... |
| 6 | 30 x 5 | 145 | 124 | .... | .... | .... |
| 7 | 20 x 5 | 14 | 11 | .... | .... | .... |
| 8 | 100 x 8 | 188 | 1 | 102 | 3 | .... |
| 9 | 50 x 17 | 9 | 1 | 36 | 9 | .... |

TABLE 5—Effect on Adult Worms of Daily Injections of Colloidal Antimony Plus Glucose (Antimoncolloid)

| Dog (No.) | First treatment for disappearance of microfilariae | | | Days from disappearance of microfilariae to second treatment | Second treatment | | | No. of days from disappearance of microfilariae to necropsy | Adult worms | | | | Toxicity of drug |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. of injections | Daily dose | Total dose | | No. of injections | Daily dose | Total dose | | Alive | | Dead | | |
| | | (mg. Sb/kg.) | | | | (mg. Sb/kg.) | | | ♂ | ♀ | ♂ | ♀ | |
| 2 | 4 | 1.5 | 6.0 | .... | .... | .... | .... | 3 | 7 | 17 | .... | .... | ± |
| 3 | 4 | 1.5 | 6.0 | .... | .... | .... | .... | 6 | 3 | 15 | .... | .... | |
| 4 | 6 | 1.5 | 9.0 | .... | .... | .... | .... | 4 | 8 | 6 | .... | .... | + |
| 7 | 2 | 3.0 | 6.0 | 15 | 3 | 1 | 3.0 | 20 | 16 | 7 | .... | .... | ++ |
| 8 | 1 | 4.5 | 4.5 | .... | .... | .... | .... | 3 | 10 | 12 | .... | 4 | ++ |
| 9 | 4 | 1.5 | 6.0 | .... | .... | .... | .... | 120 | .... | .... | .... | .... | ++ |
| 10 | 5 | 1.5 | 7.5 | .... | .... | .... | .... | 120 | 1 | .... | Several* | | + |
| 11 | 4 | 1.5 | 6.0 | 15 | 4 | 1.5 | 6.0 | 120 | 2 | .... | Several* | | ++ |
| 12 | 4 | 1.5 | 6.0 | 15 | 4 | 2.0 | 8.0 | 120 | .... | .... | Several* | | ++ |
| 13 | 6 | 1.5 | 9.0 | 15 | 6 | 1.5 | 9.0 | 120 | .... | .... | Several* | | ++ |
| 3 | 4 | 1.5 | 6.0 | 15 | 3 | 3.0 | 9.0 | 120 | .... | .... | Several* | | ++ |

* Dead worms were disintegrated in pulmonary artery; number and sex could not be determined.

sufficiently large, but if smaller doses are used, the drug must have a longer time to act.

None of the animals which received three injections of dichlorophenarsine hydrochloride in daily doses of 1.0 mg. of As/kg. showed any indication of serious intoxication, although there was occasional vomiting immediately after the injection. The vomiting was more frequent and more severe in the 2 dogs (1, 2) which received this daily dose for seven and 15 days, respectively. Severe reactions developed only in the 2 dogs (7, 9) which received single doses 3.0 mg. of As/kg. They refused to eat, had frequent and severe bouts of vomiting, and became weak rapidly. One (dog 9) died two days after treatment, but the other (dog 7) appeared to be well on the way to recovery when it was killed ten days after treatment.

## Discussion

The writer[39,43] has previously reported that nonmetallic compounds, such as acridine dyes, cyanin dyes, naphthalene, and formalin failed to injure the adult filariae. Likewise, organic compounds of mercury or bismuth have failed, in the author's experience, to injure these worms.

The evidence presented here, that the 1-diethylcarbamyl-4-methylpiperazine drugs do not kill the adult filariae, is in accord with many previously published reports.

The antimony compounds are remarkably microfilaricidal, but the effect upon the adult worms is slow and uncertain. Dead worms were not found regularly until four months after treatment. Furthermore, the doses of antimony required to effect even this delayed death of the adult worms proved toxic to all the dogs used. It is clear that such doses of these antimonials have no value in veterinary practice and can be used only in experimental studies. While antimonials clearly have no value for the destruction of the adult filariae, their possible value as microfilaricidal agents is not so sharply defined. In these experiments, a single large dose of an antimonial resulted in a rapid clearance of the microfilariae from the peripheral blood. Somewhat smaller doses may result in an initial rise in the concentration of microfilariae before their disappear-

**TABLE 6—Fate of Adult Worms in Naturally Infected Dogs at Short Intervals After Disappearance of Microfilariae Due to Daily Injection of Sodium Antimonyl Pyrogallol Sulfonate (Lyo-Antimon)**

| Dog (No.) | No. of doses | Dose (mg. Sb/kg.) Each dose | Dose (mg. Sb/kg.) Total dose | No. of days from disappearance of microfilariae to necropsy | Adult worms Alive ♂ | Adult worms Alive ♀ | Adult worms Dead | Toxicity of drug |
|---|---|---|---|---|---|---|---|---|
| 16 | 2 | 5.0 | 10.0 | 1 | 3 | 2 | .... | +++ |
| 11 | 3 | 5.0 | 15.0 | Same day | 2 | 5 | .... | ++ |
| 12 | 3 | 5.0 | 15.0 | 3 | 1 | 6 | .... | ++ |
| 13 | 3 | 5.0 | 15.0 | 5 | 5 | 1 | .... | ++ |
| 14 | 3 | 5.0 | 15.0 | 7 | 7 | 8 | .... | ++ |
| 15 | 3 | 5.0 | 15.0 | 10 | 3 | 1 | .... | ++ |

ance. Still smaller doses, comparable to those which have been used in routine practice, may produce a marked fluctuation in the numbers of microfilariae for a week or two, followed by their disappearance or reduction to a low level. When the microfilariae do not ultimately disappear as a result of the initial course of treatment, it may be difficult or impossible to destroy them with further antimony treatment. They appear to develop resistance to the antimony. Since antimonials are not well tolerated by many dogs, it is a common practice to initiate treatment with low doses and increase the dose if the animal shows no evidence of intoxication.

**TABLE 7—Fate of Adult Worms in Laboratory-Infected Dogs After Daily Injections of an Antimonial (See Table 6)**

| Dog (No.) | No. of infective larvae injected | No. of days from infection to treatment | Treatment No. of injections | Treatment Daily dose (mg. Sb/kg.) | Treatment Total dose (mg. Sb/kg.) | No. of days from treatment to necropsy | Adult worms Alive ♀ | Adult worms Alive ♂ | Adult worms Dead | Toxicity of drug |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 122 | 150 | 7 | 5.0 | 35.0 | 3 | 17 | 37 | .... | +++ |
| 2 | 100 | 150 | 5 | 5.0 | 25.0 | 130 | .... | .... | many* | +++ |
| 3 | 110 | 150 | 5 | 5.0 | 25.0 | 130 | .... | .... | many* | +++ |

* Dead worms were disintegrated in pulmonary artery; their number and sex could not be determined.

**TABLE 8—Treatment of Adult Worms with Daily Injections of Several Arsenicals**

| Dog (No.) | Name of preparations * | Daily dose (mg. As/kg.) x No. of injections | No. of days from treatment to necropsy | Adult worms Alive | Adult worms Dead |
|---|---|---|---|---|---|
| 1 | Oxophenarsine | 1 x 3 | 10 | 14 | 6 |
| 2 | Neostibarsan | 10 mg. As/kg. + 1.3 mg. Sb/kg. } x 3 | 10 | 9 | 1 |
| 3 | Thiacetarsamide | 1 x 3 | 10 | 5 | .... |
| 4 | Sodium arsanilate | 1 x 3 | 10 | 10 | .... |
| 5 | Sulfarsphenamine | 1 x 3 | 10 | 7 | .... |
| 6 | Neo-arsphenamine | 7 x 3 | 10 | 26 | .... |
| 7 | Solminol | 2 x 3 | 15 | 3 | .... |
| 8 | Solminol | 3 x 1 | 1 | 5 | .... |
| 9 | Solminol | 5 x 1 | 1 | 5 | .... |
| 10 | Carbamisin | 12 x 15 | 17 | 3 | .... |
| 11 | Filarsen | 1 mg. As/kg. + 1.5 mg. Sb/kg. } x 1 | 25 | 11 | .... |
| 12 | Daviol | 1 mg. As/kg. + 1.5 mg. Sb/kg. } x 3 | 10 | .... | Many |
| 13 | Mamol | 10 x 3 | 10 | 4 | .... |

* Common name; for tradename and formula see table 3.

**TABLE 9—Fate of Adult Worms After Daily Injections of Dichlorophenarsine-Hydrochloride**

| Dog (No.) | Daily dose (mg. As/kg.) x No. of treatments | No. of microfilariae | | No. of days from treatment to necropsy | Adult worms | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Alive | | Dead | |
| | | Pre-treatment | Post-treatment | | ♀ | ♂ | ♀ | ♂ |
| 1 | 1 x 15, v | 315 | 361 | 15 | .... | .... | 8 | 1 |
| 2 | 1 x 7, v | 5 | 17 | 15 | .... | .... | 2 | 3 |
| 3 | 2 x 2 | 75 | 55 | 7 | .... | .... | Several | |
| 4 | 1 x 3 | 488 | 493 | 15 | .... | .... | 5 | 3 |
| 5 | 1 x 3 | 7 | 10 | 10 | .... | .... | 1 | 4 |
| 6 | 1 x 3 | 255 | 118 | 10 | .... | .... | 2 | 1 |
| 7 | 3 x 1, t | 38 | 30 | 10 | .... | .... | 3 | 2 |
| 8 | 1 x 3 | 8 | 16 | 5 | 2 | 2 | .... | .... |
| 9 | 3 x 1, d | 12 | 9 | 2, d | 1 | 3 | 1 | .... |
| 10 | 1 x 2 | 49 | 37 | 30 | .... | .... | Several | |
| 11 | 1 x 2 | 75 | 55 | 20 | .... | .... | Many | |
| 12 | 1 x 2 | 35 | 2 | 20 | .... | .... | Several | |
| 13 | 1 x 2 | 115 | 121 | 15 | 1 | 1 | .... | 3 |
| 14 | 0.75 x 2 | 5 | 5 | 20 | 4 | 2 | 1 | .... |
| 15 | 1 x 1 | 61 | 104 | 30 | 1 | .... | .... | .... |

d = died of intoxication; t = severe toxicity; v = frequent vomiting.

From the standpoint of trying to clear the microfilariae from the blood stream, this may be the poorest possible procedure when using antimony. It seems clear, however, that the most valuable use of antimony in the treatment of *D. immitis* infection in the dog is for the destruction of the microfilariae. Initial heavy treatment is the most effective for this purpose.

If the antimonial acts directly on the microfilariae, it would appear probable that they would disappear more rapidly when the antimony is injected directly into the blood stream than when it is injected subcutaneously or intramuscularly. There is some suggestion in the literature [83, 88] that this is the case. However, in these experiments, the route of administration of antimony did not appear to influence the rate of disappearance of the microfilariae (table 1).

The combination of antimony and sulfonamides appears to be no more effective than antimony alone. Contrary to a previous report,[5, 35] evidence was obtained which suggested that the sulfonamides may reduce the antimony blood level and retard its activity against the microfilariae.

The organic arsenicals, unlike the organic antimonials and the nonmetallic substituted piperazine, apparently have little or no *in vivo* effect upon the microfilariae of *D. immitis*. Several of the compounds studied are well known for their therapeutic value against syphilis. However, the activity of dichlorophenarsine hydrochloride against the adult filariae appears to be a discovery. Apparently, this compound was not included in the series of arsenicals studied by Otto and Maren.[65] Three daily doses of 1.0 mg. of As/kg. are sufficient to kill all the adult worms within ten days. Even two such daily doses appear to be sufficient to kill the worms but more slowly. Such doses are practical since only minor intoxication has been observed, *i.e.*, only slight vomiting, on occasion, immediately after the administration of the drug.

Otto and Maren [61, 65] found that with thiacetarsamide the same total dose of arsenic is as effective as the dichlorophenarsine which we have used. However, thiacetarsamide must be given in 15 daily doses of not less than 0.2 mg. of As/kg., whereas, with dichlorophenarsine, three daily doses of 1.0 mg. of As/kg. is effective. Otto and Maren's only short treatment with thiacetarsamide utilized somewhat higher and more toxic doses and the dogs died three and five days after treatment, so that the worms received only a short exposure to the arsenic. It is possible that they did not adequately explore the short course of treatment with thiacetarsamide. In the studies reported here, 1 dog received exactly the same dose of thiacetarsamide (1.0 mg. of As/kg. daily for 3 days) as was found effective with dichlorophenarsine. At necropsy, ten days after treatment (when only dead worms were found after the use of dichlorophenarsine), 4 living and no dead worms were found in this animal (dog 3, table 8). This suggests that dichlorophenarsine is the more active of the two drugs. Further studies are in progress.

## Summary and Conclusions

These studies have confirmed the finding that the principal activity of the substituted piperazines (Supatonin, Hetrazan,

Caricide, and Banocide) and the organic antimonials, in filariasis, is against the microfilariae. The piperazine failed, in a limited number of dogs, to show any activity against the adult filariae. The activity of the antimonials against the adult worms was slow and uncertain and even the doses which produced delayed destruction of the worms were invariably toxic. Evidently, the only value the substituted piperazines and the antimonials have in filariasis of man or dog is in the destruction of the microfilariae.

No evidence of filaricidal activity was demonstrated for acriflavine, naphthalene, formalin, or the organic compounds of mercury or bismuth.

The marked activity of the organic arsenicals against the adult heartworm *(Dirofilaria immitis)* of the dog is fully confirmed. Dichlorophenarsine hydrochloride (Filarsen), hitherto untried against this infection, was found to be active against the adult worms and well tolerated by the dog. It appears to be more rapidly active than thiacetarsamide since two or three daily doses of dichlorophenarsine are sufficient to kill all the adult worms. Three daily doses of 1.0 mg. of arsenic (As) per kilogram of body weight will destroy the adult worms in ten days; with two such doses, their destruction appears to be slower.

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# Detection of *Anaplasma Marginale* by Means of Fluorescein-Labeled Antibody

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IDENTIFICATION of *Anaplasma marginale* in the blood and other tissues of infected cattle usually is accomplished by means of characteristic staining reactions with certain dyes. Romanowsky's stains are frequently employed for this purpose. Stained Anaplasma organisms appear in the erythrocytes as bluish purple, round marginal bodies of homogeneous dense mass, 500 mμ to 1 μ in size. While staining characteristics aid in the detection of the organism, the results obtained are often only presumptive. This is especially true in low-grade infections where only a few Anaplasma inclusion bodies are demonstrable. It is, therefore, desirable that a specific and practical method be developed for detecting *A. marginale* within tissues of infected animals. For this purpose, use of the fluorescein-labeled antibody technique of Coons *et al.*[1] was explored. The basis of the test involves immunochemical properties of the antigen-antibody complex wherein the combined fluorescent antibody renders the organism visible under the fluorescence microscope.

This report describes the use of fluorescein-labeled anti-Anaplasma globulin as a means of detecting *A. marginale* in the blood of cattle suspected of harboring the agent. In addition, the inhibitory effect of nonlabeled homologous antibody upon the reaction will be described, indicating that the method might offer important advantages in the diagnosis of anaplasmosis.

## MATERIALS AND METHODS

*Source of Anaplasma and Immune Serum.*—Anaplasmata used in this study were secured from cattle in Florida affected with acute anaplasmosis. Blood samples used for staining with labeled antibody were obtained, prior to and after splenectomy, from experimentally infected calves. The films of infected blood were prepared for staining as follows: *(a)* whole blood; *(b)* erythrocytes washed in saline solution; and *(c)* lysed erythrocytes. The antiserums were secured from experimentally infected calves at the height of infection. A number of individual antiserums, inactivated at 56 C. for 30 minutes, in which the complement-fixing antibody titers ranged from 1:80 to 1:320, were pooled and used to precipitate anti-Anaplasma globulin.

*Preparation of Anti-Anaplasma Globulin.*—A globulin fraction was prepared from the pooled antiserums by three precipitations with half saturated ammonium sulfate at 2 C. The precipitate was collected by centrifugation at 1,200 × g for 15 minutes at 4 C. The supernatant fluid was removed and the sediment dissolved in distilled water. The solution was dialyzed against 0.85 per cent sodium chloride solution at 4 C. Each milliliter of dialysate was dialyzed against approximately 333 ml. of the sodium chloride solution. Dialysing fluid was changed every six hours until the dialysate was negative for sulfate, as determined by the barium-chloride precipitation reaction. This period was usually of four days' duration. To increase the concentration of the globulin, the volume of the solution was reduced by evaporation through a dialysis sac. The final concentration of crude globulin was 1.5 Gm./100 ml. of saline solution as determined by the Biuret reaction.[2] The above solution was tested for Anaplasma complement-fixing antibodies and found positive in a dilution of 1:160.[3]

*Conjugation of Globulin Fraction.*—The globulin solution was diluted by addition of carbonate-bicarbonate buffer, pH 9, in a ratio of 10:1. The proper pH of the buffer was obtained by combining 4.4 parts of $Na_2CO_3$ with 100 parts of $NaHCO_3$, each at 0.5 M concentration. Fluorescein-isocyanate was prepared, starting with 4-nitrophthallic acid and resorcinol and following in detail the method of Coons and Kaplan.[4] Amino-fluorescein in the form of fluorescein-isocyanate was added to the globulin solution in an ice bath, and placed overnight on an agitator at 4 C. For conjugation, the proportion of 0.05 mg. of amino-fluorescein per 1 mg. of protein was used.[4] Excess fluorescein was removed by dialysing the conjugate against alkaline saline solution (pH 7.2 to 7.6) until the dialysate showed no fluorescence when examined by a Wood's lamp. The conjugate was purified by cold centrifugation at 1,000 x g for 15 minutes. Thereafter, the supernatant fluid was removed

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**TABLE 1—The Fluorescence Reaction of Bovine Blood Preparations Exposed to the Action of Various Serums**

| Types of serum | Types of cells | | | | |
|---|---|---|---|---|---|
| | Whole infected blood, 20 to 40 per cent infected cells | Washed infected cells | Lysed infected cells | Whole carrier blood with no anaplasmata demonstrable by Giemsa's stain | Whole uninfected cells |
| Immune labeled | + + + | + + + | + + + | + + | 0 |
| Normal labeled | 0 | 0 | 0 | 0 | 0 |
| Normal unlabeled followed by immune labeled | + + + | + + | + + | + | 0 |
| Immune nonlabeled followed by immune labeled | 0 | 0 | 0 | 0 | 0 |
| Immune nonlabeled and immune labeled simultaneously | + | 0 | + | 0 | 0 |
| Heterologous labeled serum | 0 | 0 | 0 | 0 | 0 |

Degree of fluorescence: + = minimal; + + = moderate; + + + = maximum.

and kept in a refrigerator for immediate use or stored at −70 C. to be used later.

*Preparation, Fixation, and Staining of Blood Films.*—A drop of the blood to be tested was placed on a glass slide and spread over an area approximately 1 cm. in diameter. The slide was dried in air at room temperature for at least 30 minutes and preserved in a refrigerator if not stained immediately. Before staining, films were fixed in absolute alcohol for ten seconds and air dried. For staining, films were treated with a drop of fluorescein-labeled anti-Anaplasma globulin and incubated at 37 C. for not less than one hour. To avoid excessive evaporation in the incubator, the preparations were placed in covered Petri dishes. Thereafter, the slides were washed in buffered saline solution, pH 7.2, for ten minutes, followed by washing in distilled water for five minutes. Films were mounted in buffered glycerol (9 parts glycerol, 1 part phosphate buffer, pH 7.0) and a cover slip was applied.

*Fluorescent Microscopy.*—A 1,000-watt, high pressure, mercury vapor bulb with a water cooling assembly, mounted in a modified Bausch and Lomb research lamp housing, was the source of light. The microscope was equipped with a cardioid dark-field condenser. The fluorescence was observed in a darkroom using a 2-mm. oil immersion objective with a 10 x ocular containing a yellow filter. Photomicrographs were taken on 35-mm. "plus X" film, using exposures of approximately three to four minutes. Intensity of the fluorescence was visually estimated and recorded as follows: 0 = no fluorescence; 1+ = low intensity fluorescence; 2+ = fluorescence of higher intensity; 3+ = very strong, sharp, yellow-green fluorescence.

*Preparation of Control-Film Specimens.*—To ascertain the immunological specificity of fluorescent staining obtained with conjugated anti-Anaplasma serum, the following control preparations were employed:

*a)* Anaplasma Plus Fluorescein-Labeled Normal Serum Globulin Reaction.—For this purpose, globulin of normal serum was employed. Since normal, labeled globulin was not expected to react with the antigen, it was anticipated that it would be removed by washing and, therefore, fluorescence would not occur.

*b)* Inhibition of the Fluorescent Staining by Unlabeled Immune Serum.—Blood films, prepared from Anaplasma-infected animals, were treated with unlabeled anti-Anaplasma serum at 37 C. for one hour. The films were washed with saline solution, after which the conjugated homologous antiserum was added and the preparations incu-

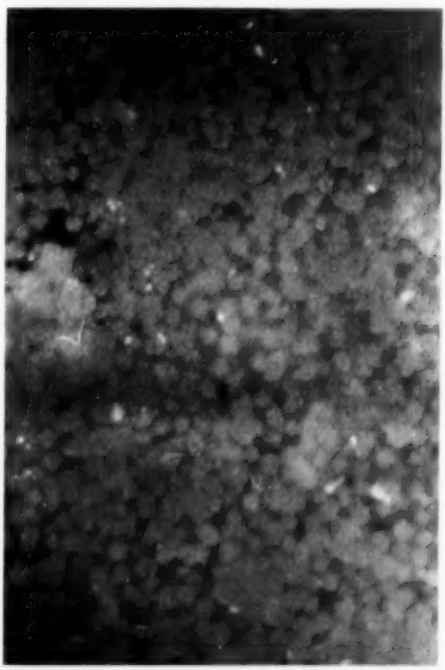

**Fig. 1—Anaplasma marginale in blood film preparation stained with homologous fluorescein-labeled antiglobulin; x 900.**

bated as above for an additional hour. Similar blood film preparations were first treated with unlabeled normal bovine serum followed by staining with labeled antibody. Specificity of the reaction was indicated if Anaplasma in the latter system demonstrated fluorescence.

## RESULTS

The results of the fluorescence reactions are summarized (table 1). Anaplasma bodies fluoresced when whole blood films from infected animals were exposed to labeled antibody (fig. 1). The anaplasmata, which occupied a marginal position with reference to the erythrocytes, appeared as brilliant, yellow-green, sharply defined, round bodies. In contrast, the erythrocytes were clearly seen as grayish green background structures. Anaplasmata did not fluoresce following exposure of similar blood films to labeled normal globulin (fig. 2). The use of washed infected erythrocytes was no more advantageous than the use of stained whole blood in these experiments.

In another trial, blood films were prepared from whole blood samples containing approximately 20 per cent infected erythrocytes as determined by the Giemsa's staining method. Lysis of the erythrocytes was accomplished by treating the films with distilled water for one minute. When the preparations were treated with homologous labeled antibody, numerous anaplasmata were demonstrated, exceeding the above reading in number (fig. 3).

The specificity of the fluorescence reaction was investigated by setting up inhibition tests as recommended by Coons and Kaplan.[2] Films of whole blood from infected animals, exposed to unlabeled anti-Anaplasma serum, were washed and treated with labeled homologous antibody. Inhibition of the fluorescence resulted (fig. 4). Application of a modified inhibition test as described by Goldman[5] was also studied. Films of Anaplasma-parasitized erythrocytes were exposed for 30 minutes to a mixture consisting of 1 drop each of labeled and unlabeled anti-Anaplasma serum. Considerable inhibition of fluorescence was observed. Specificity of the reaction was

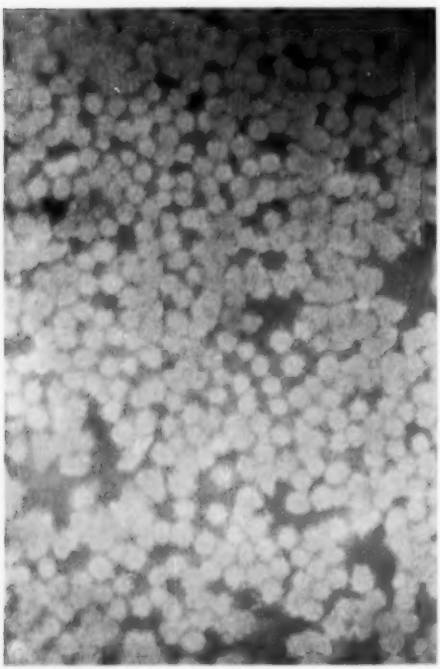

Fig. 2—Absence of fluorescence by A. marginale in a blood preparation from an infected animal when exposed to labeled normal bovine globulin; x 900.

Fig. 3—Anaplasma marginale in a water-lysed, infected erythrocyte preparation stained with homologous fluorescein-labeled antiglobulin; x 900.

further ascertained by exposing similar preparations to mixtures of labeled antibody and normal serum. No inhibition occurred in this system.

Finally, whole blood films prepared from a carrier animal, in which no Anaplasma was demonstrated by Giemsa's stain, were treated with labeled antibody. Several bodies resembling anaplasmata became fluorescent in such preparations (fig. 5).

## DISCUSSION

The advantage of the staining method described is its apparent specificity, which is based on an antigen-antibody reaction. Specific staining of Anaplasma with the labeled antibody identifies it as the actual agent responsible for the production of such antibody. In fact, specificity of the reaction now indicates that, in all probability, the Anaplasma body, located marginally with reference to the erythrocyte, represents the etiological agent of the infection and is not a manifestation of other infections or a degenerative product of the erythrocyte. Existence of interacellular Anaplasma units, in addition to the classical marginal bodies, is indicated by the fact that a greater number of fluorescent units were shown when erythrocytes were lysed prior to staining. This finding is in accordance with electron microscopic studies of Anaplasma which revealed the presence of submicroscopic bodies within the infected erythrocyte, in addition to classical Giemsa's-stained marginal Anaplasma.[7]

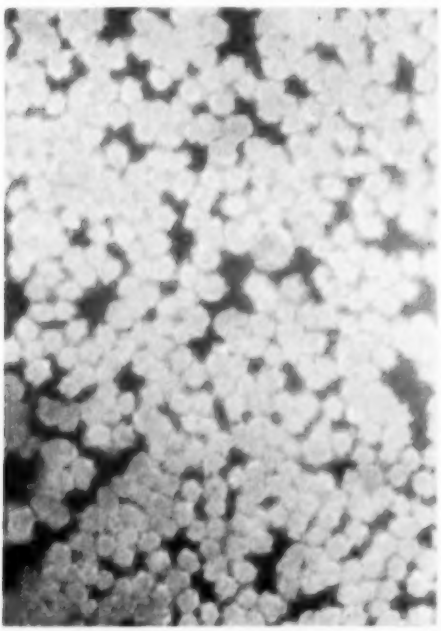

Fig. 4—Inhibition of fluorescent staining of Anaplasma marginale by treatment of film prepared from blood of an infected animal with unlabeled immune serum, followed by staining with labeled antibody; x 900.

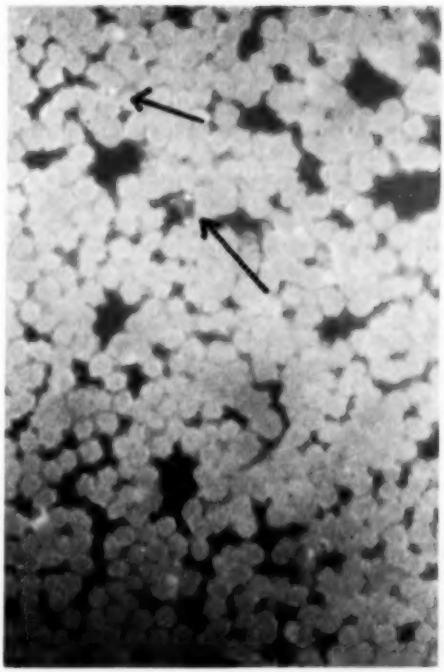

Fig. 5—Several Anaplasma marginale bodies as demonstrated in blood preparation of a carrier animal in which anaplasmata were not demonstrated by Giemsa's stain; x 900.

Studies on anaplasmata, in relation to perpetuation in arthropod vectors, have been hampered by the lack of reliable methods for detection of extra-erythrocytic forms of the organism. The method described, based on immunological specificity, offers an excellent tool in these and similar studies. Furthermore, the employment of techniques using fluorescent antibody in the study of the pathogenesis of Anaplasma is of obvious importance. For this purpose, frozen sections of organs in various stages of infection would be utilized. Such studies are now in progress.

The inhibition-of-fluorescence reaction

by unlabeled immune serums offers the possibility of utilizing such phenomena to test unknown serums for the presence of specific antibody. In fact, evaluation of individual immune serums utilizing both the complement-fixation and inhibition-of-fluorescence reactions is feasible since antibody capable of blocking the fluorescence may prove serologically different from complement-fixing antibody. The main difficulties one may encounter in establishing techniques using fluorescent antibody for diagnostic purposes would be in setting up the fluorescent microscope and obtaining the properly labeled antibody. Uniformity of the readings in various laboratories would be facilitated by obtaining labeled antibody from a reliable central source.

## SUMMARY

A globulin fraction was separated from serums of calves experimentally infected with *Anaplasma marginale* and conjugated to fluorescein. Alcohol-fixed organisms present in the infected blood films became fluorescent when exposed to the conjugated globulin. Immunological specificity of the staining was ascertained by employment of suitable controls. The ability of an unlabeled immune serum to block the fluorescence reaction offers a means of testing unknown serums for the presence of the antibody.

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# Isolation and Identification of Strychnine and Other Alkaloids in Veterinary Toxicology

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OF THE MORE common poisonous substances in animal tissues, the alkaloids are probably the most difficult to isolate and identify. A standardized procedure has been developed at this laboratory for the isolation and identification of alkaloids, particularly strychnine. It involves the extraction of the alkaloid from tissue or stomach contents by acid or an acid-alcohol mixture, preliminary testing on paper and spot-plates, paper chromatography, and ultraviolet examination.

The use of paper chromatography in the toxicological examination of alkaloids is receiving increasing consideration.[1,2,4-8] Paper chromatography lends itself to the isolation of alkaloids in low concentration, as it not only separates them in relatively pure form from interfering substances, but also partially identifies the compound. The chromatography procedures which have been advocated thus far have been of the ascending or descending types. At this laboratory, an occasion arose in which paper chromatography was needed for a large-scale separation and isolation of an unknown compound. The circular paper chromatography apparatus, as described by Giri,[3] was found to be ideal. This technique was later found to be satisfactory for the isolation and identification of strychnine in cases of canine poisoning. Additional work showed it to be ideal for the isolation and partial identification of alkaloids from animals poisoned by local toxic plants. It has the particular advantage of isolating a compound from a mixture in quantities large enough to perform additional tests for positive identification.

## MATERIALS AND METHODS

*Apparatus.*—The chromatography equipment, as described by Giri,[3] is illustrated (fig. 1). It is constructed of two thick glass plates (18 by 18 by ¼ in.) having a hole (¼ in.) at the center.

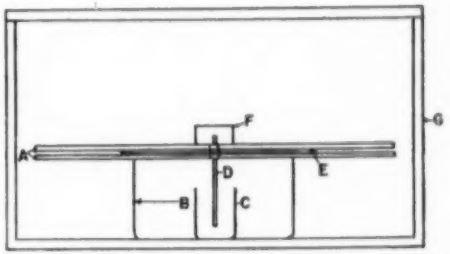


**Fig. 1—Schematic diagram of equipment: (A) plate glass, (B) support basin, (C) beaker for solvent, (D) paper wick, (E) paper, (F) glass cover, (G) enclosure box.**

The glass plates are supported by a large glass basin. Inside the basin is a small beaker which serves as a reservoir for the solvent.

Whatman No. 1 paper, 27.0 cm. in diameter, is routinely used, although paper of larger or smaller size has been used satisfactorily. Buffering of the paper was found to be unnecessary.

Spectrophotometric examination in the ultraviolet range is made by means of a Beckman model DU instrument.

*Reagents.*—Butanol-Hydrochloric Acid-Water Solvent.—Five parts of n-butanol is mixed with 1 part of concentrated HCl and the mixture is saturated with water.

Potassium Iodoplatinate.—One gram of platinic chloride is dissolved in 10 ml. of water, mixed with 250 ml. of 4 per cent potassium iodide, and water is added until a total of 500 ml. is reached.

Dragendorff Reagent.—For solution A, bismuth subnitrate (0.85 Gm.) is dissolved in 40 ml. of water and 10 ml. of glacial acetic acid. For solution B, 8 Gm. of potassium iodide is dissolved in 20 ml. of water. Before use, 5 ml. of solution A, 5 ml. of solution B, and 20 ml. of glacial acetic acid are added to 100 ml. of water.

Potassium Permanganate.—Potassium permanganate (15.8 Gm.) is dissolved in water and diluted to 1 liter.

Hydrochloric Acid, N/10.—Concentrated hydrochloric acid (8.3 ml.) is diluted to 1 liter.

Additional Reagents.—Additional reagents are sodium hydroxide, 5 per cent; tartaric acid, 10 per cent; ethyl alcohol, absolute; and chloroform.

Stock Alkaloid Solutions.—Solutions are made up containing 0.01 per cent of the alkaloid in N/10 HCl.

*Methods of Extraction.*—Tissues.—Tissue (100 Gm.) is homogenized with 50 ml. of water and 100 ml. of alcohol. The mixture is made acid to

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litmus paper with tartaric acid, refluxed for one half hour, and centrifuged while still hot. The supernatant fluid is transferred to an evaporating dish and the alcohol evaporated off on a water bath. The aqueous fraction is adjusted to pH 9.0 with 5 per cent sodium hydroxide and the supernatant fluid is transferred to a separatory funnel. Extraction is performed by vigorous shaking for five minutes, using 50 ml. of chloroform. The extraction is repeated, using 25 ml. of chloroform. If an emulsion forms, it may be broken by adding small portions of chloroform and, finally, by centrifugation. The chloroform extracts are combined, centrifuged, filtered, and then evaporated until dry. The residue is dissolved in 5 ml. of N/10 HCl and centrifuged.

It should be mentioned at this point that, although the alkaloids commonly encountered in veterinary toxicology will be extracted from a basic solution, a few may be extracted from an acid medium. With unknown alkaloids, therefore, it is recommended that the chloroform extractions be performed as described—first, from the acid solution and then from the basic solution.

Stomach Contents.—Approximately 100 Gm. of well-mixed stomach contents are homogenized with 100 to 150 ml. of N/10 HCl. The mixture is centrifuged and the supernatant fluid adjusted to pH 9.0 with sodium hydroxide. The solution is then transferred to a separatory funnel and the procedure indicated for tissues is followed.

Urine and Blood.—A measured volume of urine is adjusted to pH 9.0 with sodium hydroxide, transferred to a separatory funnel, and the procedure described for tissues is followed. The procedure for blood is the same as that for urine, except that the blood is diluted with an equal volume of water, 10 per cent trichloracetic acid added to effect protein precipitation, centrifuged, and the supernatant fluid adjusted to pH 9.0.

*Preliminary Tests.*—A preliminary test to determine if the sample extract contains an alkaloid is made by spotting a piece of filter paper with known amounts of the final acid solution. After drying, the spots are sprayed with potassium iodoplatinate, potassium permanganate, and Dragendorff reagents. The development of the characteristic color is noticed (potassium iodoplatinate: blue to blue-violet on a rose background; potassium permanganate: colorless on violet; Dragendorff: orange on yellow, which fades gradually to a yellow on white). This preliminary test also gives some indication of the concentration of alkaloid.

Another test may be made by adding a few drops of the common alkaloid-precipitating reagents (Dragendorff, potassium iodoplatinate, Wagner, phosphomolybdic acid) to 4 to 5 drops of the acid extract, mixing, and noticing the development of the precipitate. The result must be interpreted with caution since other biological compounds, such as protein-degradation products, will give a positive reaction.

*Chromatography.*—For unknown alkaloids, a circle with a diameter of 2 to 3 cm. is inscribed in the center of the paper. The unknown and standard solutions are placed on the line. The total amount of unknown solution to spot is determined by the initial trial on paper. Additional papers may be spotted with the unknown solution as described. Experience has shown that the size of the spot does not materially influence the final results. However, spotting 0.005-ml. quantities by means of a 0.1-ml. Mohr pipette, calibrated in 0.001-ml. graduations, has been found to give satisfactory spots of uniform size. The site of application is dried with a fan after each addition of solution. If the presence of strychnine is suspected, and only purification and positive identification are wanted, the solution may be spotted in the center of the paper. This allows a larger volume to be spotted and hence a greater amount of alkaloid to be separated and recovered for further testing.

A hole, the size of the paper wick, is punched through the center of the paper. The paper wicks may be made by cutting a pack of filter paper into thin strips with a paper cutter. If more than one paper is used, the spots are aligned one on top of the other and a hole punched through the pack. The paper or pack is placed between the glass plates and the paper wick inserted through the hole in the glass and allowed to extend into the beaker containing the butanol-hydrochloric acid-water solvent. Clamps may be used to tighten the glass plates together.

The solvent is allowed to advance 6 to 8 cm. from the center (approx. 2 to 3 hours for a single paper, longer as the number of papers increases). The paper is removed, the solvent front marked, and the paper allowed to dry. In the case of a paper pack, the position of each paper relative to the others is marked by cutting a notch or by making a pencil mark on the edge of each paper. A fan directed at the paper speeds the drying process. The paper is then sprayed with the developer reagents. By developing a pack of four to five papers, one developer reagent may be used per paper, or one developer on a single paper and the other papers saved for eluting the alkaloid. In those instances where the solution is spotted in the center of the paper, the paper may be cut into sectors and the individual sectors sprayed with the various reagents. The spot fronts are measured and the $R_f$ calculated from the following formula:

$$R_f = \frac{\text{measured front of unknown spot}}{\text{measured front of solvent}}$$

The position of the compound on the undeveloped papers is marked, the areas cut out into small pieces, and the alkaloid eluted with either N/10 HCl or ammoniacal chloroform. Further tests, such as color tests, microcrystallography, or ultraviolet spectrophotometry may be made on the eluate.

*Ultraviolet Examination.*—Either the initial acid extract or the acid eluate may be used. In the case of acid extracts, especially those from the stomach contents, a dilution is usually necessary to obtain satisfactory readings on the spectro-

**TABLE 1—Paper Spot Tests, $R_f$ Values, and Ultraviolet Data for Some Alkaloids**

| Alkaloid | Reagents: Potassium iodoplatinate | Reagents: Dragendorff | Reagents: Potassium permanganate | $R_f$* | Ultraviolet absorption †: Maxima |
|---|---|---|---|---|---|
| Strychnine | + | + | + | 0.78 | 254 |
| Cevadine | — | + | + | 0.69 | 261,301 |
| Hyoscyamine | + | + | + | 0.68 | ..... |
| Hysocine | + | + | + | 0.65 | 257 |
| Brucine | + | + | + | 0.67 | 265,301 |
| Monocrotaline | + | — | + | 0.65 | 203 |

* Developing solvent: n-butanol 5: HCl 1 (saturated with water). † Alkaloid in N/10 HCl.

photometer. The sample is read against the N/10 HCl as a blank. Ultraviolet absorbance is determined in the range of 220 to 340 mμ. The extinction values are plotted against wavelengths and the curve compared with curves of known alkaloids. Maximum absorbance values for some alkaloids are given (table 1). Using known alkaloid solutions, concentration versus extinction curves may be constructed and the procedure made quantitative.

*Other Identification Tests.*—The above procedures will usually suffice to identify an alkaloid. However, attention should be given to two other means of identification—color reactions and crystallography. The various procedures will be found in standard toxicology books.

## Results and Discussion

The procedure outlined above has proved satisfactory at this laboratory. The isolation procedure for tissues, etc., is not new but is an attempt to incorporate the best features of existing techniques into a method that has speed and still remains accurate. In this connection, 80 per cent recovery of strychnine added to liver tissues has been accomplished. It has been especially good in the isolation and identification of strychnine poisoning in dogs. One person can carry through a liver or stomach contents sample in approximately eight to 12 hours.

The minimum concentration of alkaloid detectable in the preliminary paper test varies with the alkaloid and with the reagent employed. Generally, 5 to 20 μg. of alkaloid can be detected. One alkaloid (cevadine, table 1) does not react with potassium iodoplatinate and another (monocrotaline) does not react with Dragendorff. From the standpoint of sensitivity, the preliminary paper spot test is recommended in place of the spot-plate test.

Stock solutions of the alkaloids strychnine, cevadine, hyoscyamine, hyoscine, brucine, and monocrotaline were chromatogrammed by the above procedure. It may be readily seen (table 1) that identification of the alkaloid solely from its $R_f$ value would not be reliable and that some confirmatory tests should be made. Chromatography is, therefore, used primarily as a rapid and easy method of purifying a compound in relatively large quantities for further testing. It is possible, however, in such cases as strychnine poisoning, to run a known alkaloid with the suspected solution and to use the resulting $R_f$ to identify the compound.

## Summary

A relatively simple and rapid procedure for isolating alkaloids from animal tissues and identifying them is described. Paper chromatography, spot-tests, and observation of absorbance in the ultraviolet range are utilized.

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# The *in Vitro* Cultivation of Tissues of Domestic and Laboratory Animals

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IN RECENT YEARS the development of tissue culture techniques suitable for use in microbiology, in general, and virology, in particular, have contributed significantly to our understanding of disease processes. The application of such systems for virus cultivation, diagnosis, and basic research to veterinary medicine is of considerable interest, provided the tissues of animals suitable for growth of the virus under investigation can be cultivated. There appears to be little doubt that many of the infectious disease entities which plague all branches of veterinary medicine are viral in origin, and knowledge regarding the etiological roles of these agents in infectious diseases of domestic mammals is inadequate. This lack of knowledge is, in large measure, due to the lack of appropriate methods with which to isolate, identify, and study material from diseases whose clinical syndrome, pathology, and epidemiology suggest viral etiology.

The success achieved by Enders *et al.*[4] in demonstrating that the poliomyelitis virus could be grown in human embryo cells, and that this growth was accompanied by "cytopathogenic changes" in those cells, placed a new tool in the hands of the virologist, with which to isolate, cultivate, and subsequently identify many viral agents. Dulbecco[6] introduced a new technique, the plaque assay system, for quantitation in animal virology. The authors' interest was thus directed toward the utilization of these newer aspects of tissue culture in veterinary medicine for isolation, identification, and mass production of virus, as well as for quantitative assay.

The literature contains few references to the *in vitro* cultivation of the tissues of domestic animals, particularly adult animals, and especially those produced as monolayer cultures as described for the monkey kidney by Dulbecco and Vogt[4] and Youngner.[20] Plasma clot cultures of canine tissue were used by Muller and Thordal-Christensen[15] and by Fieldsteel and Emery,[7] for infectious canine hepatitis virus. The latter authors also employed monolayer cultures of dog kidney. Cabasso *et al.*[2] reported the successful propagation of the same virus in roller tubes of canine kidney tissue. McClain *et al.*[14] used plasma clot cultures of swine embryonic material for the cultivation of vesicular exanthema virus, and Madin and Traum[10] reported that this virus could be propagated on monolayers of swine kidney and testicle tissues. McClain and Hackett[13] described the growth curves for vesicular stomatitis virus in a variety of monolayer systems, some derived from the tissues of domestic animals, and Bachrach *et al.*[1] reported on the growth of vesicular stomatitis virus in monolayer cultures of guinea pig kidney.

Sellers[17] grew the viruses of foot-and-mouth disease and vesicular stomatitis on monolayers of beef and swine kidney. Pay[16] described the preparation of monolayer bovine amnion, and suggested its use for viruses with definite host restrictions. Madin *et al.*[11] used monolayer cultures of beef kidney for the isolation of the virus of infectious bovine rhinotracheitis. Gustafson and Pomerat[8] have studied the cytopathogenic changes which may be present in spleen and endothelial explants from swine infected with hog cholera virus.

The literature concerning the use of tissue culture in human medicine is rapidly expanding and the reader unfamiliar with this field should consult the reviews by Enders,[5] Lynn and Morgan,[9] and also the recent papers by Weller[19] and Syverton[18] on tissue culture applications.

The present paper is a report on four years of work on the cultivation, in monolayers, of tissues of domestic and laboratory animals by a "standard method," in order that tissues suitable to veterinary virus problems would be potentially available. The system to be described permits isolation and cultivation of many viral agents, aids in their identification, and makes possible some degree of quantitation.

## MATERIALS

*Preparation of Glassware.*—Three methods of glassware preparation have been used in this laboratory over a four-year period. The last method is currently in use and has proved the most satisfactory.

1) Detergent 7x* was used in a concentration of 1 per cent in distilled water. The glassware was boiled for one hour in this solution, scrubbed, and then reboiled for an additional hour in distilled water, followed by three rinses in distilled water

From the Naval Biological Laboratory and the University of California, Berkeley.

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* Detergent 7X was obtained from the Limbro Chemical Co., New Haven, Conn.

and three rinses in deionized water. This procedure was used for ten months until difficulty was experienced, as indicated by the inconsistent growth of tissues.

2) Sodium laural sulfate in a concentration of 0.1 per cent in distilled water was used in place of the 7x. The glassware was soaked in this solution at 150 F. for one hour, scrubbed in fresh warm laural sulfate solution, rinsed in hot tap water, boiled in fresh tap water, rinsed in running tap water, followed by three rinses in distilled water and three rinses in deionized water. For approximately nine months, the glassware appeared to be satisfactory. Then erratic tissue growth again indicated that some toxic materials were present in the glassware, either as a result of accumulation of detergent, or failure to remove the toxic elements.

3) Sodium carbonate (0.1% $Na_2CO_3$ in distilled water) is the current glassware washing solution. It has proved satisfactory for the past two years, as measured by consistent tissue growth and visual cleanliness of the glassware.

All glassware is discarded into tap water immediately after use. It must be completely submerged, because if air bubbles are entrapped, rings of proteinaceous material, which are extremely difficult to remove and can cause trouble in subsequent cultures, may be left on the inside of the glassware.

Glassware is soaked for one hour at 150 F. in the sodium carbonate solution, then scrubbed and rinsed in flowing tap water. Each piece is then filled and drained several times in two separate trays of distilled water. The last rinse always contains less than 2 parts per million of ionic impurities, as measured by a conductivity meter* which reads parts per million directly.

Glassware containing petroleum base oils, or which is grossly contaminated with animal fats, should be segregated and washed separately, inasmuch as this concentration of $Na_2CO_3$ may not adequately remove these substances.

Pipettes are cleansed in concentrated sulfuric acid, thoroughly rinsed in tap water in a syphon rinser, followed by three rinses in distilled water.

All beakers, flasks, bottles, and graduates are capped with heavy aluminum foil, and all tubes are covered with aluminum tops. Pipettes, Petri dishes, and syringes are packaged in stainless steel cans or glass containers. Cotton products are not used, since they may contain harmful volatile oils. All glassware is dry air sterilized.

*Preparation of Rubber Stoppers.*—New stoppers are boiled for one hour in 0.1 per cent sodium carbonate and rinsed in three changes of distilled water. Used stoppers are discarded in tap water and need only to be boiled in distilled water, drained, repackaged and autoclaved, to be ready for use again.

Both the conventional black and white latex stoppers are satisfactory for tubes and bottles.

* Conductivity meter of Crystalab Corp., Crystal Lake, Ill.

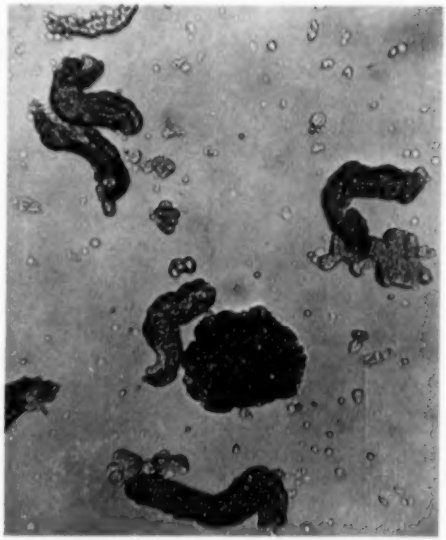

Fig. 1—Cultivable tubule suspension. x 67.

*Serums.*—Whole blood, from a pool of 10 to 20 freshly killed animals, is obtained from a local slaughterhouse and the serum harvested. Penicillin (500 μ/ml.) and streptomycin (0.5 mg./ml.) are added, and the serum is sterilized by filtration through a 2-liter Hercules† filter using a 140 mm.

Fig. 2—Noncultivable tubule suspension. x 67.

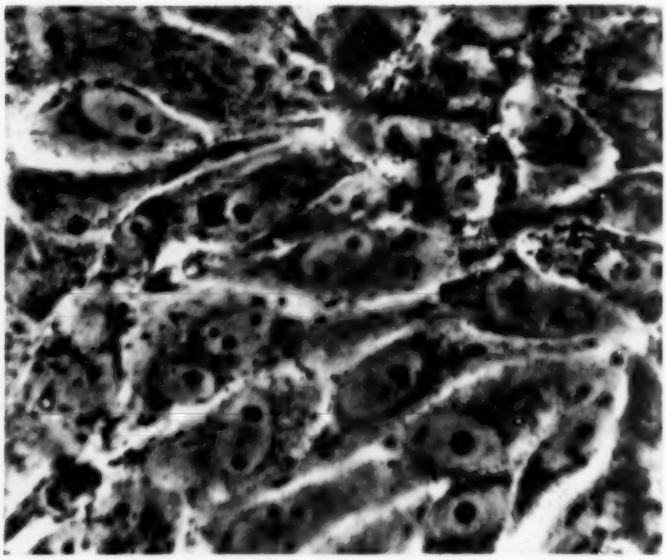

**Fig. 3—Adult swine kidney. x 210, phase contrast.**

ST-3 pad under positive air pressure. Serums are stored at ordinary refrigerator or deep freeze temperatures.

*Phosphate Buffer Solution* (PBS).—Buffer of pH 7.5, prepared by the formula of Dulbecco and Vogt,[4] is used routinely. All chemicals should be reagent grade.

Solution I

| | |
|---|---|
| NaCl | 8.00 Gm. |
| KCl | 0.20 Gm. |
| $Na_2HPO_4$ | 1.15 Gm. |
| $KH_2PO_4$ | 0.20 Gm. |
| Deionized water | 800.00 ml. |

Solution II

| | |
|---|---|
| $CaCl_2$ | 0.10 Gm. |
| Deionized water | 100.00 ml. |

Solution III

| | |
|---|---|
| $MgCl_2 \cdot 6H_2O$ | 0.10 Gm. |
| Deionized water | 100.00 ml. |

The solutions should be autoclaved separately and the fluid loss from evaporation should be replaced by adding sterile deionized water. When cool, solutions I and III are combined, followed by the addition of solution II.

*Trypsin Solution.*—The trypsin solution is prepared from 1:250 bacto-trypsin in a final concentration of 0.25 per cent by weight, using PBS as a diluent. Antibiotics (penicillin, 500 μ/ml., and streptomycin, 0.5 mg./ml.), plus 0.00125 per cent of phenol red, are added prior to filtration. The trypsin solution may be stored indefinitely at ordinary refrigerator temperature. For use it is warmed to 37 C., and maintained at this temperature during the extraction process.

† Hercules filter from Hercules Filter Corp., Paterson, N. J.

*Nutrient Mediums.*—The nutrient fluids used to date, and found most satisfactory, are: Solution M-199 of Morgan, Morton, and Parker, as commercially* prepared, and a modified lactalbumin (La) (Melnick[12]) Tris buffer† medium containing La Tris with 6 to 10 per cent serum. The formula for the latter medium is:

Solution I—Tris buffer solution

| | |
|---|---|
| Phenol red (10 mg./ml. in deionized water) | 1.0 ml. |
| Tris buffer | 2.4 Gm. |
| Deionized water | 100.0 ml. |

This makes a 0.2 M Tris solution. To bring it to pH 7.4, approximately 16 ml. of 1.0 N HCl is added.

Solution II—Modified Hank's solution

| | |
|---|---|
| NaCl | 14.35 Gm. |
| $CaCl_2$ | 0.40 Gm. |
| KCl | 0.80 Gm. |
| $MgSO_4 \cdot 7H_2O$ | 0.40 Gm. |
| $NaH_2PO_4$ (monobasic) | 0.25 Gm. |
| Dextrose | 2.00 Gm. |
| Phenol red (10 mg./ml.) | 2.00 Gm. |
| $NaHCO_3$ dissolved in $H_2O$ | 2.20 Gm. |
| Deionized water | 1700.00 ml. |

Solution III

| | |
|---|---|
| Lactalbumin enzymatic hydrolysate‡ | 10.00 Gm. |
| Deionized water | 200.00 ml. |

The lactalbumin is first mixed with water and then autoclaved at 10-lb. pressure for ten minutes. A slight precipitate, which may form as a

* M-199 solution supplied by Difco Laboratories.

† Tris buffer (Sigma 7-9) obtained from Sigma Chemical Co., St. Louis, Mo.

‡ Lactalbumin hydrolysate obtained from Nutritional Biochemical Corp., Cleveland, Ohio.

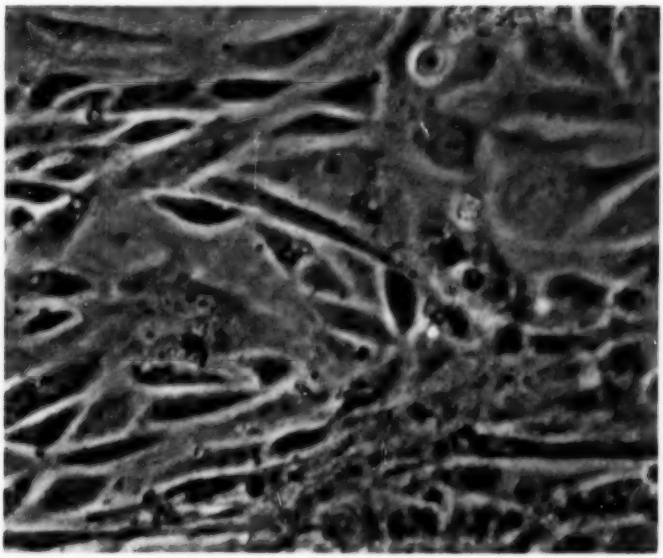

Fig. 4—Adult rabbit kidney. x 100, phase contrast.

result of autoclaving, should be disregarded. Solutions I, II, and III are combined in order, the volume made up to 2,000 ml. with deionized water, and sterilized by filtration. In practice, it has been found that both these mediums store well at ordinary refrigerator temperatures. Both the M-199 solution and the La Tris medium are fortified with 6 to 10 per cent serum prior to use. In this laboratory lamb serum is preferred, since it is readily available from young animals, and has excellent growth promoting properties for many cells.

*Antibiotics.*—Penicillin (crystalline G, 500 units/ml.) and streptomycin sulfate (0.5 mg./ml.) are added to each batch of medium prior to filtration. If the mediums are stored for more than seven days, fresh penicillin is added. To aid in the control of molds, nystatin (Mycostatin*) is added in a concentration of 50 $\mu$ per milliliter. This drug is relatively insoluble in water, but can first be put into solution in ethylene glycol (50,000 units/ml.), and then added to the mediums to give a final concentration of 50 $\mu$ per milliliter.

## Methods

*Procurement of Tissues.*—Tissues of food-producing animals are obtained from a local slaughterhouse; tissues of laboratory animals are from our own stocks. Other tissues are obtained from any available source, one of the most satisfactory being biopsy specimens.

Both adult and embryonic tissues are used. Adult tissues from food-producing animals are obtained from young animals when possible; tissues from older animals are somewhat resistant to trypsin digestion. The following age groups are used as tissue donors: swine from 3 to 8 months old, lambs from 6 to 9 months old, cattle from 8 to 16 months old, and adult horses of unknown age (probably 4 years or younger). The ages of the laboratory animals usually range from suckling to young (mice up to 8 weeks old, guinea pigs and hamsters up to 1 year old, and rabbits up to 6 months old), since these age groups are easily obtained. This does not imply that tissues from other age groups are unsatisfactory, as more mature animals have been used.

Adult tissues are usually obtained as whole organs, with no particular care exercised, except to observe a normal degree of cleanliness. Organs such as kidneys, testicles, or ovaries are removed with the capsule intact; with organs such as lungs and livers, suitable portions are excised and brought to the laboratory.

Embryonic tissues are obtained in the laboratory by removing the whole embryo from the intact uterus, usually from animals in the last trimester of pregnancy. The desired organs or tissues are removed from the fetuses using sterile instruments and by ordinary necropsy procedures. It has been our experience that rapid, skillful removal of the organs or tissues is more important to subsequent sterility and growth of the cells than is the use of sterile enclosures or complicated techniques "purportedly" designed to insure sterility.

*Preparation of Cell Groups.*—A single basic method has been used in this laboratory and represents a modification of the procedure described by Dulbecco and Vogt[4] and Youngner.[20] The technique as used for adult swine kidney fol-

* Mycostatin obtained from E. R. Squibb and Sons, New York, N. Y.

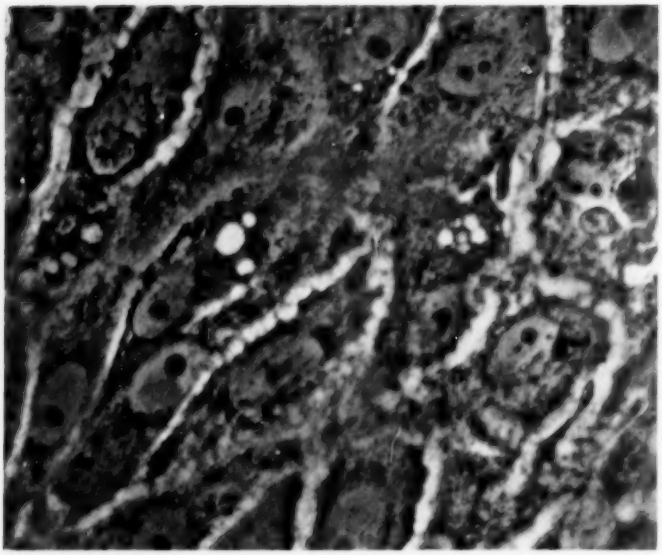

Fig. 5—Adult cat (feline) kidney. x 210, phase contrast.

lows in detail, and such modifications as are required for individual tissues are indicated.

The capsule is removed with the aid of sterile forceps and scissors. A portion of the cortex is excised (10 to 25 Gm.) and minced into pieces (3 to 5 mm.) with scalpel blades. To 1 part of tissue by weight (in an Erlenmeyer flask) 3 parts of trypsin solution by volume is added. For preliminary extraction, the kidney-trypsin mixture is agitated with a magnetic stirrer until the kidney begins to liberate tubules (approx. 30 minutes). This stage can be determined by occasional low power microscopic examination of the supernatant fluid. The appearance of cultivable and noncultivable tubules is shown (fig. 1 and 2). The supernatant fluid is discarded and fresh trypsin is added to the tissue. This mixture is then stirred by a magnetic stirrer for approximately ten minutes. The supernatant fluid is decanted and centrifuged for two minutes at 600 r.p.m.

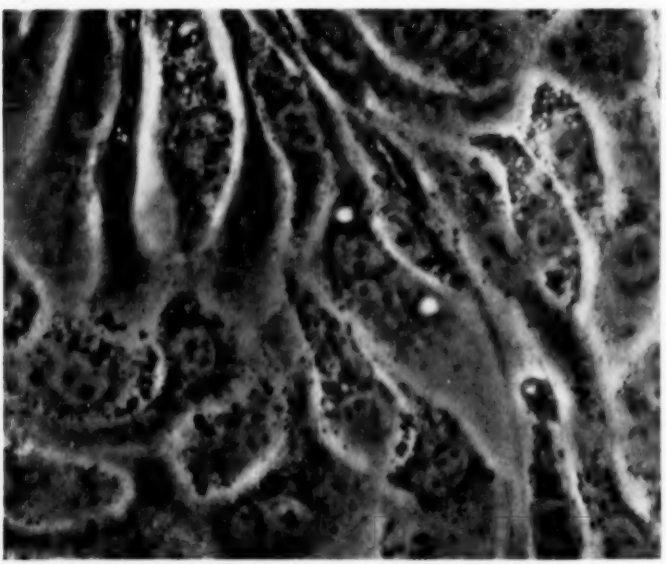

Fig. 6—Adult lamb kidney. x 210, phase contrast.

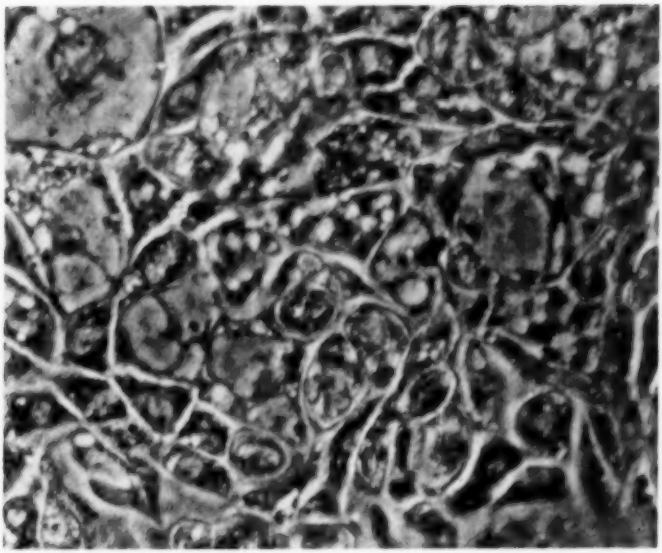

Fig. 7—Adult swine lung. x 100, phase contrast.

(6 in. radius), and the packed tubules resuspended in 10 volumes of storage fluid * at room temperature. These processing cycles are continued until the desired volume of tubules is obtained, or until the tissue is exhausted. The tubules harvested from each extraction are added to the original volume of storage fluid in a 50-ml. graduated conical centrifuge tube. When sufficient tubules have been collected, they are recentrifuged for two minutes at 600 r.p.m. The supernatant fluid is discarded and the tubules resuspended in 4 volumes of storage fluid. The tubules are then dispersed evenly throughout the fluid by gentle mixing with a pipette. The mixture is centrifuged as described above, the volume of packed tissue is measured, the supernatant fluid is discarded,

* Nutrient mediums, with or without serum, and Dulbecco buffer have been used individually as storage fluid at various times. All seem to be satisfactory.

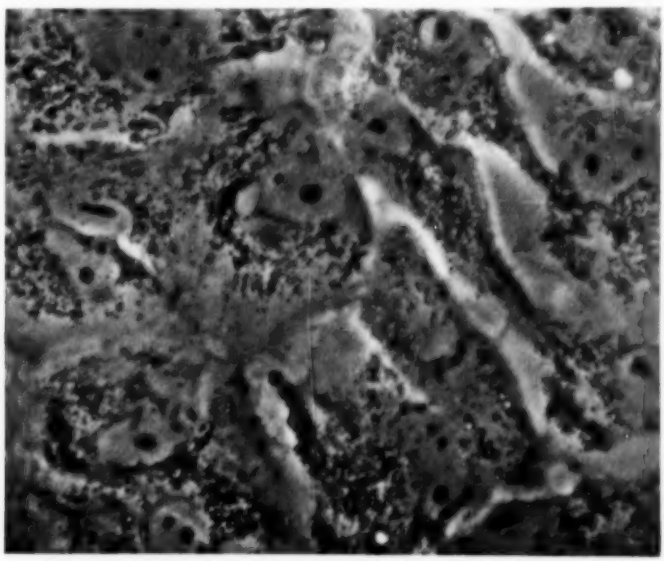

Fig. 8—Swine embryonic kidney. x 210, phase contrast.

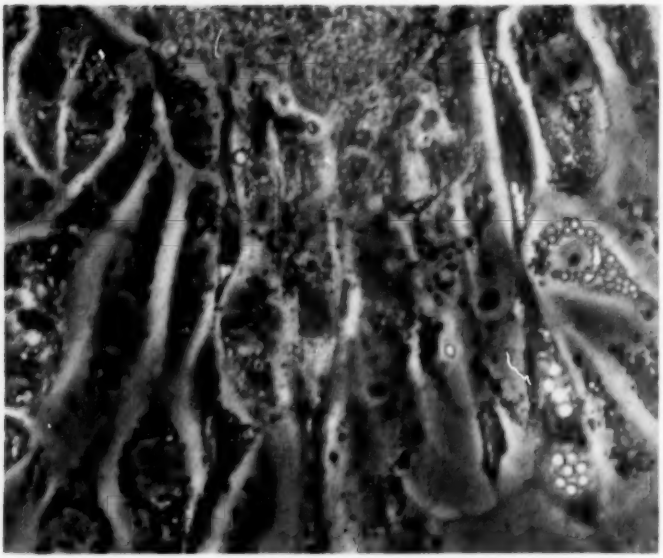

Fig. 9—Swine embryonic lung. x 100, phase contrast.

and the cells dispersed in 4 volumes of nutrient fluid.

The dispersed cells are transferred to a large Erlenmeyer flask which contains the volume of nutrient fluid necessary to complete a 1:200 dilution. The cells are then dispensed into tubes, using a Cornwall syringe; or into bottles of various sizes, either using a pipette or pouring directly. During the dispensing procedure, the flask containing the cells is repeatedly agitated to minimize settling or clumping. One milliliter of tubule suspension is used for a standard culture tube (150 by 15 mm.), 10 ml. for a milk dilution bottle, 80 ml. for a Blake bottle, 6 ml. for a small Petri plate (60 mm.), and 12 to 15 ml. for the large Petri plate (100 mm.).

Incubation is carried out without motion at 37 c. Bottles are laid flat in trays, while tubes are inclined at a slight angle. Petri plates are maintained in a flowing atmosphere of air and co$_2$

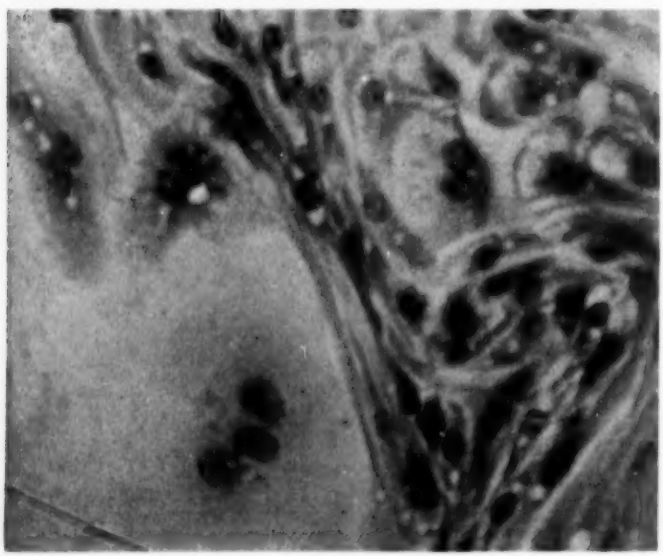

Fig. 10—Amnion, swine cells. Note multinucleated cell. Giemsa's stain. x 210.

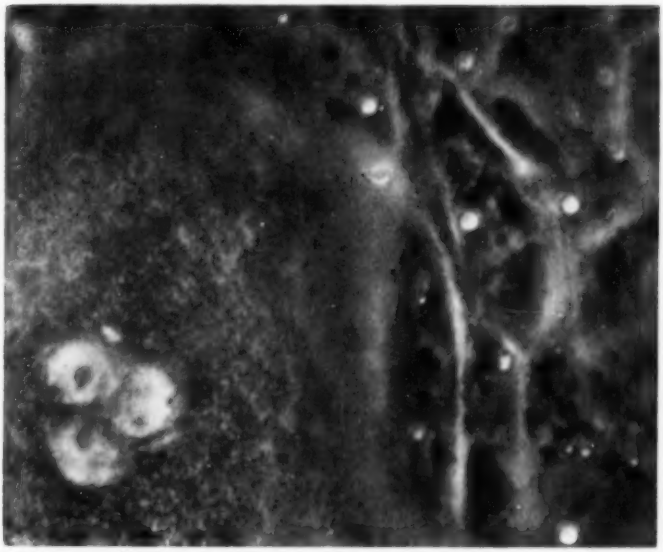

Fig. 11—Amnion, swine cells. Note multinucleated cell. x 210, phase contrast.

(approx. 4%), adjusted to maintain a pH of 7.2 in the medium covering the cells.

Cultures are left undisturbed for 48 hours, after which time they are usually "stuck" to the glass and beginning to multiply. They may now be examined macroscopically for changes in pH and microscopically, under low power (100 x), for evidence of cell growth. The old nutrient medium is always replaced after 48 to 72 hours, and again as needed in about six to seven days. Usually, the pH of an individual culture will indicate the necessity of a fluid change, since a marked shift to the acid side brings about a yellow color in the fluid, indicating that fresh fluid is desirable. When adequate growth has occurred (7 to 12 days), the cultures are ready for use.

Embryonic tissues are prepared by the same method. In practice, minced embryonic tissues can be left in contact with the trypsin solution overnight at room temperature, thus facilitating extraction procedures the following morning. Once a work routine has been established, 1,000 tubes of a given culture can be prepared in a four-hour period by two experienced operators.

Certain tissues have been found to require minor deviations from the general techniques described. These include: (1) For bladder, the mucosa must be washed extensively with PBS to remove all traces of urine. Small portions of the epithelial mucosa are removed from the submucous layer, minced into fragments of 3 to 5 mm., and then processed as described, except that the supernatant fluid from the extractions is centrifuged at 1,000 r.p.m., instead of 600 r.p.m. (2) Lung tissue is obtained from the margin of the lung to minimize the amount of large bronchiole tissue, and is then cut into large pieces (25 to 100 mm.) for digestion. These large pieces prevent accidental decanting of the floating tissue fragments after each cycle. (3) For skin, the epithelial layers are removed from embryonic skin by manually peeling this layer from the underlying layers. (4) Cervix tissue requires extensive washing of the mucosa to remove the mucus which is usually present. The mucosa can then be removed by careful stripping.

The remaining tissues cultivated to date have presented no particular problems that could not be solved within the outline of the general method.

## Results and Discussion

The results of attempts to cultivate a large variety of both adult and embryonic mammalian cells in monolayer cultures are shown (table 1), and selectively illustrated (fig. 3–10). The number and variety of tissues cultivated from various domestic and laboratory animals indicate that the described methods are satisfactory, and that success can be anticipated if they are properly carried out. For purposes of this study, "growth" designates not only the fact that the tissues grow in a monolayer on the glass, but that sufficient growth is produced for virus inoculation or for extended observation of the growth characteristics of the cells. As indicated (table 1), tissues occasionally fail to grow. In certain instances, this failure may be ascribed to technical difficulties; in others, however, the reason is not understood. Experi-

ence has shown that, despite the currently available methods, a small percentage of tissues from individual animals do not grow, and that such failures are to be expected from time to time.

The cell types which propagate from the various tissues are of interest. In most instances, this method of cultivation gives rise to mixed cell populations, usually composed of epithelial or parenchymal cells, typical of the particular organ, and fibroblasts. Kidney cultures yielded predominantly tubular epithelium and fibroblasts (fig. 3). Testicle cultures yielded a mixture of Sertoli cells and fibroblasts. Bladder cultures consist of pure transitional epithelium, while cultures of skin and cervix appear to be statified squamous epithelium. The lung cultures are composed of fibroblasts plus cells derived from the alveolar walls (fig. 7). Liver cultures produce pure liver parenchyma cells. Amnion cell cultures are characterized by the presence of large multinucleated cells (fig. 10) in addition to the mononuclear elements.

Embryo cultures yielded cells that varied in proportion to the differentiation of the various organ systems cultured. In cultures derived from very young embryos, it was extremely difficult to identify the individual cells. Cultures from the differentiated organs, such as lung, liver, and kidney, of older embryos, give rise to cells similar in appearance to those derived from similar adult organs.

Even though many of the organs produce characteristic cells, readily recognizable in young, actively growing cultures, the ability to define the cell types precisely is dependent upon such variables as culture, age, and nutrition. Young, actively growing cultures usually exhibited the cellular detail and over-all pattern characteristic of a particular organ. Older cultures often assumed the appearance of fibroblasts, either as a result of overgrowth by existing fibroblasts, or as a result of a form of metaplasia to cells resembling fibroblasts. Under some nutritional influences, apparently adverse, the cells may swell, become granular and distorted in shape, and even develop vacuoles in their cytoplasm. Such cells will often return to their normal

**TABLE 1—Results of the Attempted in Vitro Cultivation of a Variety of Adult and Embryonic Mammalian Tissues in Monolayers**

| Species | Tissue | Adult growth/attempts | Embryonic growth/attempts |
|---|---|---|---|
| Cattle | Kidney | 13/13 | 5/5 |
| | Testicle | 1/1 | ... |
| | Cervix | 1/1 | ... |
| Dog | Kidney | 1/1 | ... |
| Horse | Kidney | 11/11 | ... |
| | Tongue | ... | 2/2 |
| | Sarcoid (neoplasm) | 2/2 | ... |
| Cat | Kidney | 14/14 | ... |
| Guinea pig | Kidney | 3/4 | 1/3 |
| Guinea pig, suckling | Kidney | 2/4 | ... |
| Hamster | Kidney | 6/7 | 3/3 |
| | Uterus | 1/1 | ... |
| Hamster, suckling | Kidney | 3/5 | ... |
| Lamb | Kidney | 7/7 | 1/1 |
| | Liver | ... | 1/1 |
| | Testicle | 4/5 | ... |
| | Brain | ... | 1/1 |
| Monkey | Kidney | 3/4 | ... |
| | Spleen | 1/1 | ... |
| Mouse | Mammary tumor * | 3/5 | ... |
| Mouse, suckling | Kidney | 15/18 | ... |
| | Brain (whole) | 5/5 | ... |
| Rabbit | Kidney | 1/1 | ... |
| Rabbit, suckling | Kidney | 8/8 | ... |
| Swine | Amnion | 10/11 | ... |
| | Bladder | 2/2 | ... |
| | Cornea | ... | 1/1 |
| | Iris | ... | 1/1 |
| | Kidney | 98/118 | 21/26 |
| | Liver | 1/2 | ... |
| | Lung | 5/6 | 2/2 |
| | Ovary | 1/1 | ... |
| | Sarcoma | 1/1 | ... |
| | Testicle | 23/33 | ... |

* Adenocarcinoma.

appearance as a result of a change in environment.

The life span of the various cultures has not been investigated in detail, but experience has indicated that the majority of cells, if properly cared for, will continue to grow vigorously for at least three to four months. Adult swine kidney has been preserved in excellent condition for as long as 15 months, and there is no reason to believe that this time period could not be extended. In this laboratory, the majority of the cultures have been used for virus production or assay within the first two weeks of their existence.

## SUMMARY

The method described provides for the *in vitro* cultivation of a large number of tissues, from a variety of mammalian hosts, which are suitable for the isolation, growth, and assay of many viral agents.

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# *Clostridium Botulinum* Type C. I. Studies on Laboratory Variables Affecting Toxin Production and Toxoid Evaluation

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IN RECENT YEARS, workers in several countries have reported on the preparation of *Clostridium botulinum* type C toxoid for immunization of cattle and mink.[1,2,5,10,16] The problems of types C and D intoxication in cattle and sheep are confined primarily to South Africa and Australia. In South Africa, the disease is known as "carrion disease" or "lamziekte."

Dinter and Kull[6] reported food poisoning on 11 mink ranches in Sweden during 1949 and 1950, and were able to identify the toxin as type C on three of the farms. While they were unable to positively diagnose botulism on the remaining ranches, the disease was presumptively diagnosed as such, and several cogent reasons were given for not making a positive identification. The mortality ranged from 2 to 100 per cent. Kull and Moberg[5] determined that botulism was the cause of 13 of the 22 cases of food poisoning in mink which they studied, and that type C toxin was responsible in 12 of the 13. Mortality ranged from 4.6 to 100 per cent. Quortrup and Gorham[12] earlier demonstrated the high relative susceptibility of mink to type C botulinum toxin.

Sterne and Wentzel[16] described the large-scale preparation of *Cl. botulinum* types C and D toxoids using the dialysate system previously described by Polson and Sterne,[12] Gladstone,[7] and others. Bell *et al.*[2] described the production of a trivalent toxoid composed of equal parts of culture of types A, B, and C. They showed that mink could be immunized against more than 100,000 mouse lethal doses of type C toxin by using a 1-ml. dose of the toxoid. They did not challenge with types A and B toxin.

Larsen *et al.*[10] reported the successful immunization of 56 mink with type C dialysate toxoid against a challenge dose of approximately 10,000 mouse lethal doses. Appel *et al.*[1] described the immunization of 643 mink with two type C toxoid preparations, one of which was an alumina gel adsorbate, while the other was "precipitated" with potassium aluminum sulfate, and showed good protection with the alumina gel adsorbate toxoid. The mink were protected against a challenge of $10^{5.9}$ mouse lethal doses of type C toxin. The results with the alum-precipitated toxoid were poor, presumably because of a previously unrecognized low titer of the parent toxin.

There is little information concerning *Cl. botulinum* type C in literature, and the majority of reports from the field, both in this country and abroad, have incriminated type C in outbreaks on mink ranches. It was felt that more basic information was needed regarding type C toxins and toxoids prior to actual testing of toxoids under field conditions.

## MATERIALS

*Strains.*—Eight strains were studied. Strain 526, isolated in 1920 from a horse, was received from Dr. K. F. Meyer, University of California, Berkeley. Strains X-220B-1, 6060-A, 6060-B, and 462 were sent by Dr. J. Frederick Bell, National Microbiological Institute, Rocky Mountain Laboratory, Hamilton, Mont. Strain X-220B-1 was originally isolated from a duck liver and was reportedly of high toxigenicity. Strains 462, 6060-A, and 6060-B were atoxigenic. Strains 6605 and South Africa (SA) were received from Dr. Austin E. Larsen, Rocky Mountain Fur Breeders' Association, Salt Lake City, Utah, and strain 468 from Dr. E. L. Mundell, Jensen-Salsbery Laboratories, Kansas City, Mo.

After initial identification procedures and verification of type with type C-specific antiserum* by toxin neutralization in healthy white mice, three toxigenic strains (SA, X-220B-1, and 468) and one atoxigenic strain (6060-A) were selected for further study.

*Mediums.*—After screening several mediums, two were determined to be most suitable for growth and toxin production. One, hereafter referred to as SW medium, was similar to the medium described by Sterne and Wentzel,[16] and contained filtrate of alkalinized (pH 8.6) corn step liquor (Staley) equivalent to 5.0 per cent solids; $CaCl_2 \cdot 2H_2O$ (0.1%); final pH 7.4 after autoclaving. The second, hereafter called FD medium, was described by Cardella *et al.*,[4] and contained protease peptone (4%); enzymatic hydrolysate of casein (2%); yeast extract (2%); dextrose (1%); final pH 7.6.

*Toxin Titration.*—In most instances, titrations were conducted in healthy white mice obtained from the same source. The mice were of the same strain and sex (male), and ranged in weight from 15 to 25 Gm. All toxin dilutions were made in phosphate buffer (pH 6.5) containing 0.2 per cent gelatin.[11] With one exception, whole culture toxins

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* Supplied by Dr. Arden Killinger, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa.

were used. The l.d.$_{50}$ end points were calculated by the method of Reed and Muench.[14]

## METHODS AND RESULTS

*Preliminary Toxoid Evaluations.*—Toxoids were prepared from 5- to 10-day cultures by adding 0.5 per cent formalin and reincubating at 37 C. for eight to 12 days. Both toxigenic strain SA and atoxigenic strain 6060-A were used as seed inoculums. A toxoid was prepared from the latter, since Boroff[3] reported the production of high-titer antiserums in rabbits after immunization with an atoxigenic strain of *Cl. botulinum* type C. The toxoids were adsorbed in a manner similar to that described by Rice *et al.* [15] and were subsequently diluted in physiological saline solution. Mice were immunized intraperitoneally with 0.5-ml. doses of toxoid. Where two doses were used, the second followed the first by three days. At 14 days after the first dose, all mice were challenged intraperitoneally with approximately 33 m.l.d. of cell-free toxin contained in 0.1 ml. of saline solution (table 1).

It was obvious that toxoids prepared from the atoxigenic strain 6060-A did not protect mice, and that two doses of the toxoid prepared from the SA strain stimulated a more effective immunity than did a single dose.

*Comparison of Toxin-Production Methods.*—The efficacy of the mediums described above in supporting growth and toxin production of *Cl. botulinum* type C was determined both with and without the dialyzing membrane assembly. The l.d.$_{50}$ of dialysate and nondialysate toxins produced in each medium is shown (table 2).

**TABLE 2—Titers of Dialysate and Nondialysate Toxins in Mice***

| Medium | Culture system | Calculated l.d.$_{50}$ |
|---|---|---|
| SW | Dialysate | $10^{-8.0}$ |
| SW | Nondialysate | $10^{-5.25}$ |
| FD | Dialysate | $10^{-8.50}$ |
| FD | Nondialysate | $10^{-6.25}$ |

* Intraperitoneal injection.

The immunogenicity of toxoids prepared from each of the four toxins was then evaluated in mice. The toxoids were prepared as previously described. Where one dose of toxoid was used, the mice were given 0.5 ml. intraperitoneally; where two doses were given, a second similar dose followed the first by three days. The results of intraperitoneal challenge at 14 days after the first dose are shown (table 3).

Table 3 indicates that the toxoid prepared from the FD-dialysate system stimulated the best immunity response in mice. Toxin yields from this system have been consistently higher than those from other methods studied. Observations during several phases of this study indicated that toxin produced in the SW medium, and particularly in nondialysate cultures, was less stable than that produced in the FD-

**TABLE 1—Challenge of Mice Immunized with Initial Preparations of Adsorbed Clostridium Botulinum Type C Toxoid**

| Strain | Toxoid dilution | No. doses toxoid | Total dose (ml.) | Dead/inoc. (48 hours) | Protected (%) |
|---|---|---|---|---|---|
| South Africa (toxigenic | None | 1 | 0.5 | 0/5 | 100 |
| | 1:2 | 1 | 0.5 | 3/5 | 40 |
| | 1:4 | 1 | 0.5 | 2/5 | 60 |
| | 1:8 | 1 | 0.5 | 3/5 | 40 |
| | 1:16 | 1 | 0.5 | 3/4 | 25 |
| | None | 2 | 1.0 | 0/4 | 100 |
| | 1:2 | 2 | 1.0 | 0/3 | 100 |
| | 1:4 | 2 | 1.0 | 0/5 | 100 |
| | 1:8 | 2 | 1.0 | 1/5 | 80 |
| | 1:16 | 2 | 1.0 | 3/4 | 25 |
| 6060-A (atoxigenic) | None | 1 | 0.5 | 5/5 | 0 |
| | 1:2 | 1 | 0.5 | 5/5 | 0 |
| | 1:4 | 1 | 0.5 | 5/5 | 0 |
| | 1:8 | 1 | 0.5 | 5/5 | 0 |
| | 1:16 | 1 | 0.5 | 5/5 | 0 |
| | None | 2 | 1.0 | 4/4 | 0 |
| | 1:2 | 2 | 1.0 | 5/5 | 0 |
| | 1:4 | 2 | 1.0 | 5/5 | 0 |
| | 1:8 | 2 | 1.0 | 4/4 | 0 |
| | 1:16 | 2 | 1.0 | 4/4 | 0 |

dialysate cultures. This may account, in part, for the poor immunogenicity of the toxoids prepared from toxin produced in the SW medium.

*Variables.*—The influence of injection route, dosage volume, and interval between immunization and challenge on the immunity response in mice were studied as variables in routine laboratory immunization schedules. FD-dialysate toxoid was used in this study. The challenge results are shown (tables 4 and 5).

There appeared to be little difference in effectiveness between intraperitoneal and subcutaneous routes of immunization. The difference in results obtained with one as compared with two immunizing doses was not considered significant. The threshold of effective immunity in mice appeared to be 0.5 ml. whether the toxoid was administered in one or two doses. Below this volume, with the toxoid preparations used, immunity began to drop off rapidly.

## DISCUSSION

*Other Observations.*—Of the several strains of *Cl. botulinum* type C studied, only three were determined to be toxigenic for the white mouse. Strains 6060-A, 6060-B, and 462 were reportedly atoxigenic and appeared to be so. Strains 6605 and 526 were supposedly toxigenic, but proved to be otherwise in these studies. Strains SA, X-220B-1, and 468 proved to be highly toxigenic. The titers were consistently between $10^{5.5}$ and $10^{6.5}$ l.d.$_{50}$ per milliliter in white mice. The toxin produced in the mediums studied with strains X-220B-1 and 468 appeared to be more unstable than that produced by strain SA. Lyophilized whole cultures of the three toxigenic strains have been routinely used in this laboratory for seed and for standard cultures.

Lamanna *et al.*[9] concluded that the lethality of botulinum toxins (types A, B, and C) "... bears neither a simple nor proportional relationship to the body weight of white mice. ..." Male mice weighing 6 to 10 Gm. are killed more rapidly than females of the same weight or heavier mice of either sex, according to Lamanna, but since the rapidity of death is not the important criterion in most immunization studies, the significance as applied to immunization is lost.

Several mediums were investigated for optimum growth and toxin production. The corn steep medium of Sterne and Wentzel [16] gave inconsistent toxin yields and resulted in a toxin of less stability than that produced in the FD medium. Sterne and Wentzel [16] indicated that glucose in the growth medium inhibited toxin production, and Boroff [3] suggested that the presence of 0.2 per cent glucose in the beef heart infusion (Difco) used in his medium

**TABLE 3—Immunity Stimulated in Mice by Dialysate and Nondialysate Toxoids**

| Culture | Toxoid dilution | Doses | Total dose (ml.) | Challenge dose l.d.$_{50}$* | Dead/inoc. (10 days) | Protection % |
|---|---|---|---|---|---|---|
| SW dialysate | 1:5 | 1 | 0.5 | 32 | 0/4 | 100 |
| | 1:10 | 1 | 0.5 | 32 | 1/4 | 75 |
| | 1:5 | 2 | 1.0 | 3,200 | 3/5 | 40 |
| | 1:10 | 2 | 1.0 | 3,200 | 5/5 | 0 |
| SW nondialysate | 1:5 | 1 | 0.5 | 32 | 5/5 | 0 |
| | 1:10 | 1 | 0.5 | 32 | 4/4 | 0 |
| | 1:5 | 2 | 1.0 | 3,200 | 5/5 | 0 |
| | 1:10 | 2 | 1.0 | 3,200 | 4/4 | 0 |
| FD dialysate | 1:5 | 1 | 0.5 | 32 | 1/4 | 75 |
| | 1:10 | 1 | 0.5 | 32 | 0/3 | 100 |
| | 1:5 | 2 | 1.0 | 3,200 | 0/5 | 100 |
| | 1:10 | 2 | 1.0 | 3,200 | 0/5 | 100 |
| FD nondialysate | 1:5 | 1 | 0.5 | † | † | † |
| | 1:10 | 1 | 0.5 | † | † | † |
| | 1:5 | 2 | 1.0 | 3,200 | 2/4 | 50 |
| | 1:10 | 2 | 1.0 | 3,200 | 2/4 | 50 |

* All mice were challenged intraperitoneally.

† All mice died after vaccination, before time of challenge. Sterility checks on this preparation in ten days indicated that the organisms were killed but that the toxin was not detoxified. On previous and subsequent occasions, similar preparations were not detoxified in 14 days at 37 C. with 0.5 per cent formalin, probably because of the higher proportion of extraneous proteinaceous material present, which resulted in greater binding of the formalin.

**TABLE 4—The Influence of Route of Immunization and Interval Before Challenge on Immunity Response in Mice**

| Group | Challenge dose* 14 days (l.d.$_{50}$) | Challenge dose* 21 days (l.d.$_{50}$) | Dead/inoc. (4 day) | Protected (%) |
|---|---|---|---|---|
| 1 dose (0.5 ml.) intraperitoneally | 42,200 | ..... | 0/5 | 100 |
| | 4,220 | ..... | 2/5 | 60 |
| | ..... | 31,600 | 0/5 | 100 |
| | ..... | 3,160 | 1/5 | 80 |
| 2 doses (each 0.5 ml.) intraperitoneally | 42,200 | ..... | 0/5 | 100 |
| | 4,220 | ..... | 0/5 | 100 |
| | ..... | 31,600 | 0/5 | 100 |
| | ..... | 3,160 | 0/5 | 100 |
| 1 dose (0.5 ml.) subcutaneous | 42,200 | ..... | 2/5 | 60 |
| | 4,220 | ..... | 0/5 | 100 |
| | ..... | 31,600 | 0/5 | 100 |
| | ..... | 3,160 | 1/5 | 80 |
| 2 doses (each 0.5 ml.) subcutaneous | 42,200 | ..... | 2/5 | 60 |
| | 4,220 | ..... | 0/5 | 100 |
| | ..... | 31,600 | 0/5 | 100 |
| | ..... | 3,160 | 0/5 | 100 |
| Controls | Dilution | Dilution | | Mortality † (%) |
| 14-day challenge | $10^{-4}$ | ..... | 5/5 | 100 |
| | $10^{-5}$ | ..... | 5/5 | 100 |
| | $10^{-6}$ | ..... | 1/5 | 20 |
| Controls 21-day challenge | ..... | $10^{-4}$ | 5/5 | 100 |
| | ..... | $10^{-5}$ | 4/5 | 80 |
| | ..... | $10^{-6}$ | 1/5 | 20 |

* All mice were challenged intraperitoneally.

† The l.d.$_{50}$ of the toxin used for the 14-day challenge was $10^{-5.625}$; the l.d.$_{50}$ of the toxin used for the 21-day challenge was $10^{-5.50}$.

may have favored the formation of atoxigenic variants. In these studies, there were no indications that glucose inhibits toxin formation, and Cardella *et al.*[4] suggest that the addition of glucose may, in fact, be beneficial.

Toxoids prepared from nondialysate systems were, on the whole, unsatisfactory. Low-titer toxins of greater lability were, in general, obtained from the nondialysate systems. The immunizing ability of botulinum toxoid, as might be expected, appears to be critically dependent upon the presence of high-titer stable toxin in the parent culture. Several studies in this laboratory have shown that toxoids prepared from FD-dialysate cultures, when properly adsorbed, will consistently protect mice against more than 40,000 l.d.$_{50}$. What this represents in terms of immunity engendered in mink will be reported in a subsequent paper correlating the immunity response of mice and mink to *Cl. botulinum* type c toxoids.

It was observed early in these studies that the type c toxin was unstable when handled improperly. Rice *et al.*[15] reported that after filtration through paper pulp and Mandler cultures, type A cultures suffered a loss of toxicity for mice and guinea pigs of from 50 to 75 per cent. We have observed a drop in toxicity for mice, with type c cultures, of approximately 60 per cent after filtration through Selas No. 2 candles and Seitz EK filters. These observations tend to support the hypothesis of Boroff[3] that the toxic fraction is intimately associated with the bacterial cytoplasm and, thus, when intact cells are filtered out a severe drop in toxicity results. The classical distinction between exotoxins and

**TABLE 5—The Influence of Dosage Volume on Immunity Response in Mice**

| Total toxoid dose (ml. per mouse*) | Challenge dose l.d.$_{50}$ | Dead/inoc. (4 day) | Protection (%) |
|---|---|---|---|
| 0.2 | 320 | 2/3 | 33.3 |
| 0.4 | 320 | 1/4 | 75.0 |
| 0.6 | 320 | 0/4 | 100.0 |
| 0.8 | 320 | 0/5 | 100.0 |
| 1.0 | 320 | 0/5 | 100.0 |

* The mice were immunized with two doses of toxoid, the second following the first by three days. Each dose was one half the total volume.

endotoxins may be more artificial than previously assumed.

## SUMMARY

Several factors concerned with the preparation and laboratory evaluation of *Clostridium botulinum* type c toxoids were studied:

1) Under the conditions of these experiments, strain South Africa (SA) appeared to produce more stable toxin than did other toxigenic strains.

2) Formalinized whole-culture suspensions of atoxigenic strain 6060-A, incorporated with an aluminum adsorbate, were nonimmunogenic in mice.

3) In general, toxins produced in dialysate systems had higher titers and yielded more immunogenic toxoids than did toxins obtained from nondialysate systems.

4) For toxin and toxoid preparation, a medium similar to that described by Cardella *et al.*[4] was found to be more satisfactory than a modification of the Sterne and Wentzel [16] medium.

5) Mice were found to be excellent experimental animals for evaluating type c botulinum toxoid preparations.

6) The subcutaneous and intraperitoneal routes were found to be equally effective for immunizing mice.

7) A 14-day interval between vaccination and challenge was apparently sufficient to demonstrate the full immunity response in mice.

8) With the toxoid preparations used, the threshold of immunity response in mice appeared to be 0.5 ml. whether the toxoid was given in one or two doses.

Toxoids that protect mice against more than 40,000 lethal doses of type c botulinum toxin have been prepared by the methods and criteria described.

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# A New Species of Brucella Isolated from the Desert Wood Rat, *Neotoma Lepida* Thomas

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IN A RECENT REPORT, by Bendtsen *et al.*,[1] natural infection of hares with *Brucella suis* and the epizootiological relationship between leporine and porcine brucellosis in Denmark were described. In their paper, the literature on leporine brucellosis in Europe was rather completely reviewed. Other workers[2-4] have recovered Brucella from rats which were collected on premises where brucellosis was prevalent in cattle. The purpose of this report is to describe a new species of Brucella isolated from the desert wood rat, *Neotoma lepida* Thomas, a rodent whose habitat is almost completely isolated from domestic livestock. The name, *Brucella neotomae*, is proposed for this organism.

## MATERIALS AND METHODS

Collections of *N. lepida* were made in the Great Salt Lake Desert in Utah. Specimens were taken alive in traps, bled, and were immediately frozen after euthanasia. Carcasses were transported to the laboratory under dry-ice refrigeration and held at −20 C. until thawed preparatory for examination. Pooled triturated tissue suspensions, consisting of portions of the lungs, liver, spleen, and kidneys from 1 to 4 rats, were cultured by surface plating of 0.1 ml. of each suspension on a tryptose agar plate.[5] After four days of aerobic incubation at 37 C., the plates were examined for typical colonies.

Essentially, procedures recommended in the "Manual of Methods for Pure Culture Study of Bacteria"[6] were employed in the identification of isolates. However, certain exceptions are noted. For fermentation studies, a broth base (pH 7.3) of the following composition was used: bacto-tryptose, 10.0 Gm.; sodium chloride, 5.0 Gm.; di-sodium phosphate, 1.0 Gm.; and distilled water, 1,000.0 ml. Seitz-filtered substrates were added to make a 1 per cent concentration and phenol red was used as an indicator. Initial fermentation tests were conducted in 16- by 150-mm. tubes containing 10.0 ml. of medium.

To further evaluate the changes in pH which occurred in dextrose, galactose, levulose, arabinose, and xylose, fermentation reactions were repeated in 20- by 150-mm. tubes containing 15.0 ml. of medium. For each strain, three tubes of each sugar were inoculated with a 2-mm. loopful of a heavy suspension of organisms. At four, eight, and 14 days postinoculation, the pH of unaltered living cultures was determined by an electropotentiometer. Uninoculated tubes of base with substrates and base without sugars, incubated under the same conditions, were used as controls.

Hydrogen sulfide production during growth on tryptose agar was measured daily with strips of filter paper impregnated with lead acetate. *Brucella abortus* (strain 19-26A), *Br. suis* (strain 6), and *Brucella melitensis* (strain 16M) were used as controls in comparative studies of antigenic content and biochemical reactions. Before use in various studies, strains of *Br. neotomae*, and the above three species of Brucella were determined to be smooth by the acriflavine test.[7] In all tests, 48- to 72-hour growth on tryptose agar was used for inoculum.

Sensitivity to aniline dyes was evaluated by the method recommended by Huddleson.[8] Dilutions of 1:25,000, 1:50,000 and 1:100,000 of thionin (C. I. 920, certification NF16, 86% dye content) and basic fuchsin (C. I. 677, certification NF62, 95% dye content) were used. Actual dye content was used as a basis for calculating dilutions.

The antigenic composition of *Br. neotomae* (5K33) was compared with that of *Br. abortus* and *Br. melitensis* by the cross-absorption agglutination test. Since *Br. suis* is antigenically identical with *Br. abortus* when compared by this test, it was deemed unnecessary to include the former in this experiment. Antigens used in this study were prepared according to the following procedure: A 72-hour growth on tryptose agar was removed by washing with 0.5 per cent formalized saline. After a slow centrifugation which removed bacterial clumps and particles of agar, the density of each antigen was adjusted so that 0.3 ml. of stock suspension diluted in 10.0 ml. saline gave 60 per cent light transmittance when measured in a Klett-Summerson colorimeter equipped with a No. 42 filter. Antiserums against each organism were prepared by immunization of rabbits with two 1.0-ml. doses of a milky suspension of viable cells given intravenously at a seven-day interval; rabbits were then exsanguinated two weeks after the second injection. Aliquots of two-fold dilutions of each antiserum were mixed with

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an equal quantity of each of the three antigens and incubated for four hours at 37 C. and then overnight at 4 C. After agglutinated bacteria were removed by centrifugation, the absorbed serum was tested for residual agglutinins.[9]

To determine susceptibility to infection with *Br. neotomae*, white Swiss mice, Rocky Mountain Laboratory strain, and guinea pigs, procured from local breeders, were inoculated intraperitoneally with decimal dilutions of a suspension of cells. Six weeks after inoculations, animals were bled, killed, and a triturated pool of lungs, liver, kidney, and spleen of each animal was cultured on tryptose agar.

## RESULTS

Five strains of *Br. neotomae* were recovered from 36 pools of tissue from *N. lepida*, representative of 108 animals. These isolates are designated as follows: 5K33, 5E1169, 5E1266, 6D152, and 5G239. The serological status of the animals had not been determined at the time when the majority were subjected to necropsy. Hence, cultures were made from aliquots of suspension frozen at the time they were originally tested for various other pathogens. However, at the time specimen 5K33 was examined, agglutinins against Brucella had already been detected in the serum of this animal. From organs of this rat, cultured individually, an isolation was made only from the liver.

The organism grows aerobically and increased $CO_2$ tension is not required for primary isolation. Maximum growth on agar slants is obtained at 37 C. incubation; growth is delayed at 30 C., and at 42 C. growth is inhibited. Very little growth occurs on tryptose agar incubated anaerobically and, in agar shake cultures, growth is restricted to the medium within 2 mm. of the surface. On tryptose agar plates inoculated with infectious tissue suspensions, growth appears after incubation for three days. After six days of incubation, isolated colonies may reach a diameter of 3 mm. Colonial morphology of this organism is similar to that of other Brucella. Colonies are convex, amorphous, smooth, glistening, and their edges are entire. Young colonies are semitransparent and appear uniformly opalescent when viewed with transmitted light, whereas older colonies develop distinctly brown centers.

The newly isolated Brucella is a nonmotile coccobacillary organism. In cultures grown on tryptose agar for three days, cells are approximately 0.6 $\mu$ in diameter by 0.6 to 1.4 $\mu$ in length. It is gram-negative and, like other Brucella, the organism also retains the basic fuchsin of Macchiavello stain. No flagella, capsule, or endospore could be demonstrated with recommended stains.[6]

*Brucella neotomae* does not produce indol and is negative to the Voges-Proskauer and methyl red tests; nitrates are reduced to nitrites. Gelatin is not liquefied and litmus milk remains unchanged. With Bauer's urease test,[10] all five strains are positive after ten minutes of incubation. Maltose, sucrose, lactose, raffinose, salicin, melezitose, rhamnose, trehalose, inulin, inositol, sorbitol, mannitol, and glycerol are not fermented.

A moderate amount of acid without gas was produced in galactose, dextrose, and

**TABLE 1—Comparison of pH Changes Produced in Selected Carbohydrates by Three Species of Brucella and Five Isolates of Brucella Neotomae**

| Brucella organism | pH of mediums after indicated days of incubation at 37 C. | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Galactose | | | Levulose | | | Xylose | | | Dextrose | | | Arabinose | | | Base without carbohydrate | | |
| | 4 | 8 | 14 | 4 | 8 | 14 | 4 | 8 | 14 | 4 | 8 | 14 | 4 | 8 | 14 | 4 | 8 | 14 |
| *Br. abortus* (19-26A) | 7.3 | 7.2 | 7.1 | 7.3 | 7.4 | 7.4 | 7.3 | 7.2 | 7.3 | 7.3 | 7.3 | 7.4 | 7.2 | 7.1 | 7.0 | 7.4 | 7.6 | 7.8 |
| *Br. suis* (6) | 7.3 | 7.4 | 7.5 | 7.4 | 7.4 | 7.6 | 7.2 | 7.2 | 7.0 | 7.3 | 7.4 | 7.4 | 7.2 | 6.9 | 6.6 | 7.5 | 7.7 | 7.8 |
| *Br. melitensis* (16M) | 7.3 | 7.3 | 7.4 | 7.3 | 7.4 | 7.5 | 7.3 | 7.3 | 7.4 | 7.4 | 7.5 | 7.6 | 7.2 | 7.0 | 6.9 | 7.4 | 7.7 | 7.9 |
| *Br. neotomae* (5K33) | 6.9 | 6.5 | 6.2 | 7.0 | 6.9 | 6.7 | 6.9 | 6.5 | 6.4 | 6.9 | 6.7 | 6.4 | 6.8 | 6.5 | 6.0 | 7.3 | 7.4 | 7.5 |
| *Br. neotomae* (5E1169) | 6.8 | 6.4 | 6.2 | 7.0 | 6.8 | 6.6 | 6.8 | 6.6 | 6.4 | 6.9 | 6.6 | 6.4 | 6.8 | 6.4 | 5.9 | 7.4 | 7.4 | 7.5 |
| *Br. neotomae* (5E1266) | 6.9 | 6.6 | 6.2 | 7.0 | 6.8 | 6.7 | 6.8 | 6.6 | 6.4 | 6.9 | 6.6 | 6.5 | 6.7 | 6.4 | 6.0 | 7.4 | 7.4 | 7.5 |
| *Br. neotomae* (6D152) | 6.8 | 6.4 | 6.2 | 7.0 | 6.8 | 6.7 | 6.8 | 6.6 | 6.5 | 6.9 | 6.7 | 6.5 | 6.8 | 6.4 | 5.9 | 7.4 | 7.4 | 7.5 |
| *Br. neotomae* (5G239) | 6.8 | 6.4 | 6.1 | 7.0 | 6.8 | 6.6 | 6.8 | 6.5 | 6.4 | 6.9 | 6.6 | 6.4 | 6.8 | 6.5 | 6.0 | 7.4 | 7.4 | 7.5 |
| Uninoculated controls | 7.2 | 7.2 | 7.2 | 7.2 | 7.0 | 6.9 | 7.2 | 6.9 | 6.8 | 7.2 | 7.2 | 7.1 | 7.1 | 7.0 | 6.9 | 7.3 | 7.3 | 7.3 |

**TABLE 2—Comparison of the Sensitivity of Five Isolates of Brucella Neotomae with the Sensitivity of Typical Species of Brucella to Thionin and Basic Fuchsin**

| Brucella organism | Thionin | | | Basic fuchsin | | |
|---|---|---|---|---|---|---|
| | 1:25,000 | 1:50,000 | 1:100,000 | 1:25,000 | 1:50,000 | 1:100,000 |
| *Br. abortus* (19-26A) | 0 | 0 | 0 | +* | +++ | ++++ |
| *Br. suis* (6) | ++++ | ++++ | ++++ | 0 | 0 | 0 |
| *Br. melitensis* (16M) | 0 | 0 | ++ | +++ | ++++ | ++++ |
| *Br. neotomae* (5K33) | 0 | 0 | 0 | 0 | 0 | 0 |
| *Br. neotomae* (5E1169) | 0 | 0 | 0 | 0 | 0 | 0 |
| *Br. neotomae* (5E1266) | 0 | 0 | 0 | 0 | 0 | 0 |
| *Br. neotomae* (6D152) | 0 | 0 | 0 | 0 | 0 | 0 |
| *Br. neotomae* (5G239) | 0 | 0 | 0 | 0 | 0 | 0 |

* + indicates amount of growth.

arabinose (table 1). Although the final pH of levulose and xylose at 14 days after inoculation with *Br. neotomae* was only slightly lower than that in uninoculated tubes of control sugars, the pH of these two sugars inoculated with three known species of Brucella was significantly higher. The final pH of sugar-free, tryptose base inoculated with three recognized species of Brucella was more alkaline than the same medium inoculated with *Br. neotomae*; this increased alkalinity may possibly reflect differences in the ability to deaminate amino acids in the base.[11] With the exception of arabinose inoculated with *Br. suis*, the pH of other sugars inoculated with the three recognized species of Brucella remained above the pH values of uninoculated control sugars.

Production of $H_2S$ on tryptose agar by strains of *Br. neotomae* was similar to that of *Br. suis* (fig. 1). Slightly less $H_2S$ was formed by *Br. neotomae* during the first 24-hour period but, thereafter, the amount and duration of $H_2S$ production was essentially the same as that of *Br. suis*.

Results of dye sensitivity tests (table 2) were obtained with freshly isolated cultures of *Br. neotomae*. These cultures were inhibited by dilutions (1:100,000) of thionin and basic fuchsin that are usually recommended for the typing of Brucella. However, the sensitivity of subcultures of strains 5G239 and 5K33 changed during cultivation on tryptose agar for five and nine months, respectively. After such prolonged cultivation on artificial medium, these subcultures were partially inhibited by a dilution of 1:50,000, but they were not inhibited by a 1:100,000 dilution of thionin.

The results of cross-absorption tests (table 3) clearly indicate a close antigenic relationship between *Br. abortus* and *Br. neotomae* and a dissimilarity between these two and *Br. melitensis*. Antibodies in appropriate dilutions of *Br. neotomae* antiserum were completely removed by absorption with *Br. abortus* antigen and vice versa. However, agglutinins in *Br. melitensis* antiserum were not completely removed by absorption with *Br. abortus* or *Br. neotomae* antigens.

Based on limited titrations, the intraperitoneal m.i.d.$_{50}$ for mice was determined to be two to three organisms, whereas a comparable dose for guinea pigs was about a hundredfold greater. The organism was regularly recovered from mice and guinea pigs for 42 days after inoculation. After 60 days postinoculation, some guinea pigs had cleared their tissues of the organism but others remained infected for 150 days, the longest interval tested. Gross pathological changes in infected mice and guinea

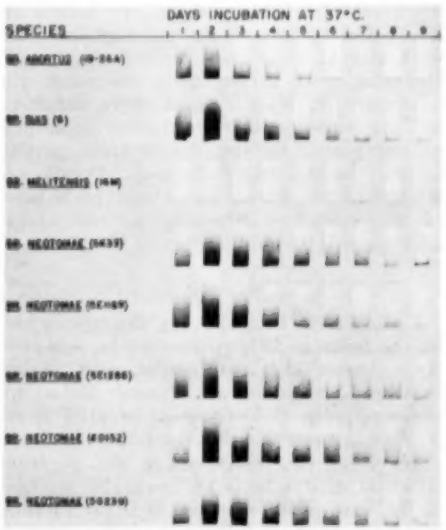


**Fig. 1—Comparison of the $H_2S$ production of five isolates of Brucella neotomae with that produced by recognized species of Brucella.**

**TABLE 3—Cross Absorption of Brucella Melitensis, Brucella Abortus, and Brucella Neotomae Antiserums with Their Respective Antigens**

| Antiserum | Dilution | Absorbed with: | Residual agglutinins† against: | | |
|---|---|---|---|---|---|
| | | | A | B | C |
| *Br. melitensis* | 1:4 | A* | 16 | 32 | 32 |
| *Br. melitensis* | 1:8 | A | 0 | 0 | 0 |
| *Br. melitensis* | 1:16 | A | 0 | 0 | 0 |
| *Br. melitensis* | 1:4 | B | 32 | 64 | 32 |
| *Br. melitensis* | 1:8 | B | 32 | 0 | 0 |
| *Br. melitensis* | 1:16 | B | 16 | 0 | 0 |
| *Br. melitensis* | 1:4 | C | 64 | 16 | 32 |
| *Br. melitensis* | 1:8 | C | 16 | 16 | 16 |
| *Br. melitensis* | 1:16 | C | 16 | 0 | 0 |
| *Br. melitensis* | 1:8 | Saline | 128 | 128 | 128 |
| *Br. abortus* | 1:8 | A | 0 | 16 | 32 |
| *Br. abortus* | 1:16 | A | 0 | 32 | 32 |
| *Br. abortus* | 1:32 | A | 16 | 32 | 32 |
| *Br. abortus* | 1:8 | B | 0 | 16 | 32 |
| *Br. abortus* | 1:16 | B | 0 | 0 | 0 |
| *Br. abortus* | 1:32 | B | 0 | 0 | 0 |
| *Br. abortus* | 1:8 | C | 0 | 32 | 32 |
| *Br. abortus* | 1:16 | C | 0 | 0 | 0 |
| *Br. abortus* | 1:32 | C | 0 | 0 | 0 |
| *Br. abortus* | 1:16 | Saline | 128 | 64 | 128 |
| *Br. neotomae* | 1:2 | A | 16 | 128 | 256 |
| *Br. neotomae* | 1:4 | A | 64 | 128 | 128 |
| *Br. neotomae* | 1:8 | A | 0 | 32 | 32 |
| *Br. neotomae* | 1:2 | B | 0 | 16 | 16 |
| *Br. neotomae* | 1:4 | B | 0 | 0 | 0 |
| *Br. neotomae* | 1:8 | B | 0 | 0 | 0 |
| *Br. neotomae* | 1:2 | C | 0 | 16 | 32 |
| *Br. neotomae* | 1:4 | C | 0 | 0 | 0 |
| *Br. neotomae* | 1:8 | C | 0 | 0 | 0 |
| *Br. neotomae* | 1:8 | Saline | 128 | 64 | 128 |

* A = *Br. melitensis*; B = *Br. abortus*; C = *Br. neotomae*. † Reciprocal of titer of absorbed serum dilution.

pigs were minimal. Although testicular abscesses were occasionally observed, multiple necrotic foci, such as are found in livers and spleens of guinea pigs infected with any of the three common species of Brucella, were infrequently observed. In immature *N. lepida* which were infected by ingestion or intraperitoneal injection of organisms, no significant gross pathological changes were observed. Details of studies on the pathogenesis and pathology of *Br. neotomae* infections in laboratory animals will be published later.[12]

## DISCUSSION

Cultures of Brucella are usually typed on the bases of $CO_2$ requirements, sensitivity to dyes, $H_2S$ production, and agglutination in absorbed antiserums. Based on these criteria, the organism isolated from *N. lepida* more closely resembles *Br. suis* than either *Br. melitensis* or *Br. abortus*. *Brucella neotomae* is antigenically similar to *Br. suis* or *Br. abortus*, $H_2S$ production is similar to that of *Br. suis*, and increased $CO_2$ tension is not required for primary isolation, but *Br. neotomae* is inhibited by concentrations of thionin which do not affect *Br. suis*.

An outstanding characteristic which distinguishes *Br. neotomae* from the three common Brucella is the ability of the former to ferment certain carbohydrates. Acid production in xylose, arabinose, glucose, levulose, and galactose by five isolates of *Br. neotomae* was uniform. Changes in pH in the above sugars differed significantly from those produced by typical species of Brucella. In addition to the three strains of common Brucella listed (table 1), two typical strains of *Br. abortus* and two typical strains of *Br. suis* were tested for their ability to ferment carbohydrates. With exception of the production of acid in arabinose by the two strains of *Br. suis*, the pH of inoculated sugars after 14 days of incubation was consistently more alkaline than that of uninoculated controls.

The greater infectivity of *Br. neotomae* for mice than for guinea pigs also distinguishes this organism from other Brucella. Although available information on the susceptibility of white mice to infection with Brucella is rather meager, it is generally accepted that guinea pigs are more susceptible than mice.[13,14]

Since colonial and cellular morphology, antigenic composition, and most of the biochemical properties of *Br. neotomae* are similar to those of *Br. abortus* or *Br. suis*, it is illogical to assign the organism recovered from *N. lepida* to a genus other than Brucella. Fermentation reactions in dextrose, levulose, xylose, arabinose, and galactose; dye sensitivity pattern; and comparative infectivity for mice and guinea pigs differentiate *Br. neotomae* from the three common species of Brucella.

## SUMMARY

1) Five strains of an organism, for which the name *Brucella neotomae* is proposed, were recovered from *Neotoma lepida* collected in Utah.

2) Characteristics of these strains were compared with properties of three recognized species of Brucella.

3) Colonial and cellular morphology, antigenic composition, and most of the biochemical properties of *Br. neotomae* are similar to some members of the genus Brucella; however, the isolate from *N. lepida* produces acid without gas in dextrose, levulose, xylose, arabinose, and galactose; is sensitive to both thionin and

basic fuchsin; and is more infective for mice than for guinea pigs.

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# Propagation of Virus Diarrhea Virus of Cattle in Tissue Culture

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THE ORIGINAL publications by Olafson and co-workers,[1,2] and subsequently by Baker and his co-workers,[3] have shown that virus diarrhea of cattle is a clinical entity which is caused by a specific virus. The concentration of virus in the cattle tissues that were tested was uniformly low. In this work, the effects and concentration of virus in bovine embryonic tissues were studied.

## MATERIALS AND METHODS

*Virus Used.*—The cattle passage of strain 1 virus diarrhea isolated in New York State[3] was used in this study. A 10 per cent suspension of spleen from an infected animal was made with a glass grinder and 0.2 ml. used to initiate transfers of virus in bovine embryonic skin-muscle tissue using the chicken clot method.

To simplify the procedure and to observe effects in another tissue prepared in a different manner, 0.2 ml. of undiluted fluid phase from the twentieth transfer of virus propagated in embryonic skin-muscle tissue was inoculated into tubes containing embryonic bovine kidney cortex prepared after the manner of Dulbecco and Vogt.[4]

*Method of Culture.*—Roller tube cultures were prepared with minced fragments of skin-muscle tissue embedded in clotted chicken plasma after the method of Enders.[5] Skin-muscle tissue was obtained from bovine embryos 1 to 3 months of age. Seven to 14 fragments introduced into each tube provided satisfactory growth of cells. The first medium used to propagate the cells was a fluid composed of bovine amniotic fluid (90%), inactivated horse serum (5%), and bovine embryo extract (5%). Phenol red in a concentration of 15 mg./1,000 ml. was added. Penicillin and streptomycin were added so that the final concentration was 100 units and 100 μg. per milliliter, respectively. Fluid was changed at two- to four-day intervals. In this medium, luxurious cell growth was achieved. The cultures obtained in this medium were used for all transfers except 12 to 17. For these transfers, the medium consisted of Earle's balanced salt solution (89.5%), lactalbumin (0.5%), and inactivated horse serum (10%) with 100 units of penicillin and 100 μg. of streptomycin per milliliter. Although satisfactory cell growth was achieved in this medium, the extent of growth was less than that in the first medium. After inoculation of virus, tubes were placed in a roller drum for incubation at 37 C. Serial transfers of virus to fresh tissues were made at intervals ranging from seven to 14 days by inoculation of 0.2-ml. amounts of supernatant fluid that had been pooled and centrifuged.

The trypsinization method of Dulbecco and Vogt was used to prepare the embryonic bovine kidney cortex cells. The embryos were 1 to 7 months old. The same medium used for passages 12 to 17 of skin-muscle, tissue-cultured virus was also used to initiate growth of cells at 37 C. After five to seven days, the fluid for maintenance of cells was replaced with the same medium, except 1 per cent inactivated horse serum was used instead of 10 per cent. One series of virus transfers in cultured cells was made at five-day intervals by inoculation of 0.2 ml. of centrifuged supernatant fluid that had been pooled from four tubes, representing twentieth virus transfer in skin-muscle cells. Another series of similar virus transfers was made at eight-day intervals. The tubes were placed in roller drums or tissue culture racks for incubation at 37 C. On occasions, it has been necessary to store the tissue-cultured virus under dry ice refrigeration.

*Tests for Virus.*—The fluid phase of the eleventh virus transfer produced in skin-muscle, tissue-cultured cells was tested undiluted and the twentieth passage was tested in dilutions of $10^{-2}$ to $10^{-5}$ in cattle. One calf was inoculated intravenously with 1 ml. of eleventh transfer and 2 calves were inoculated intravenously with 1 ml. from each dilution of twentieth transfer.

The fluid phase of fifth virus transfer in bovine kidney cells that had been incubated for eight days was diluted 1:100 and 1 ml. was inoculated into 1 calf. The fluid phase of the tenth virus transfer in bovine kidney cells incubated for eight days, and of the fifteenth virus transfer in bovine kidney cells incubated for five days, were titrated in cattle. The dilutions inoculated ranged from $10^{-5}$ to $10^{-7}$ with tenth transfer and from $10^{-6}$ and $10^{-7}$ with fifteenth transfer. One milliliter of each dilution was inoculated into 1 animal.

Preinoculation blood serum samples were obtained from all test calves and daily total leukocyte counts were made on each test calf. Temperatures were taken twice daily and each animal was observed for signs of illness. After 14 days, each animal was bled for convalescent serum and inoculated intravenously with 1 ml. of a 10 per cent suspension of spleen taken from a calf show-

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ing typical signs of illness.[1-3] Daily examinations were made for signs of illness and temperatures were taken twice a day for two weeks.

## RESULTS

Virus diarrhea virus has been carried for 20 serial transfers in skin-muscle tissue culture and 15 additional transfers in embryonic bovine kidney cells. During the course of this study, cultures inoculated with virus have been examined and no cytopathogenic effects in cells were observed in either type of tissue culture system.

The calf inoculated with eleventh tissue culture, fluid passage, showed signs of illness. The calves that were inoculated with the $10^{-3}$, $10^{-4}$, $10^{-5}$, and $10^{-6}$ dilutions of the twentieth tissue-cultured virus showed signs of illness as did 1 of 2 calves given $10^{-7}$ dilution; the other, and the 2 calves given the $10^{-8}$ dilution, did not show signs of illness. When given cattle test virus 14 days later, none of the calves that reacted to tissue-cultured virus showed signs of illness, indicating that they were immune, while the calves that showed no signs of illness after injection with the tissue-cultured virus dilutions sickened after inoculation with cattle test virus.

The calf given the 1:100 dilution of fifth virus transfer in bovine kidney cells showed the usual signs of illness. The tenth virus transfer that was incubated for eight days produced signs of illness in the calf given the $10^{-5}$ dilution of tissue-cultured fluid. The $10^{-6}$ and $10^{-7}$ dilutions elicited no reaction. The fifteenth transfer in bovine kidney cells incubated for five days produced signs of illness in the calf given the $10^{-6}$ dilution, but not in the calf given the $10^{-7}$ dilution. None of the calves that reacted to tissue-cultured virus showed signs of illness when given cattle test virus 14 days later, proving that they were immune. The calves that displayed no signs of illness after injection with tissue-cultured virus dilutions became ill when injected with cattle test virus.

## DISCUSSION

The evidence found by these experiments indicates that the virus of virus diarrhea reproduced itself *in vitro* when cultivated in suitable tissue cells and medium. The final dilution of the original material was so great as to rule out conclusively the presence of original virus. The virus propagated in cultures retained its virulence for cattle, for when it was inoculated intravenously, typical illness followed.

It seems to be of significance that the concentration of virus in tissue-cultured virus ranged from $10^5$ to $10^7$. In contrast, the concentration of virus in tissues from infected animals usually was $10^3$ to $10^4$. The reason for this difference is not clear at present, but may be answered by further studies.

Since the concentration of virus in cultures of bovine embryonic tissue is as high as $10^7$, it may be possible to produce suitable antigens in tissue culture for use in serological studies. The need for a serological test to study this disease and its prevalence is great. This is especially true now, since there is so much confusion surrounding the so-called "virus diarrhea-mucosal disease" complex of cattle in the United States.

## SUMMARY

A strain of virus diarrhea virus isolated from cattle in New York State has been maintained for 20 consecutive passages in bovine embryonic skin-muscle tissue prepared in roller tubes by the plasma-clot method and for 15 further transfers in embryonic bovine kidney cortex cells prepared by trypsinization. Multiplication of virus was not associated with conspicuous cytopathogenic effect on cells, but virus apparently was maintained at full virulence. The concentration of virus in fluid from these tissue-cultured cells ranged from $10^5$ to $10^7$.

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# Infection of Laboratory Mice with *Salmonella Kentucky*

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EPIZOOTICS of Salmonella infection in colonies of laboratory mice are of frequent occurrence and of considerable concern to the breeder and to the laboratory worker. Two Salmonella species are commonly incriminated in the etiology of mouse typhoid: *Salmonella typhimurium* and *Salmonella enteritidis* (Gaertner and Danysz varieties). This is a report of a sporadic case of infection of a laboratory mouse with a different Salmonella species, *Salmonella kentucky*, never hitherto isolated from mice.

A female albino mouse, strain CFW, and her 7-day-old litter of 6 were found dead five days after the suckling mice had received an intraperitoneal inoculation of a human stool specimen which was free of bacterial pathogens. Necropsy of the adult mouse revealed the presence in the liver of a large number of white, circular lesions, 1 to 2 mm. in diameter, with a grayish center. The lesions were found in the whole liver parenchyma as well as on its surface. They were not observed in any other organ of the adult mouse nor in the livers of the suckling mice. Plating on horse blood agar of a suspension of the adult mouse liver led to the isolation of two types of colonies: one type was found to be an organism of the Proteus group, and the other one, predominant in number, was identified further as *S. kentucky* (antigenic formula: [VIII], XX: i — $z_{16}$).*

*Salmonella kentucky* has been isolated in the past from the intestinal tract of a chick,[1] during an outbreak of enteric infection in camels in Israel,[2] from a human case of gastroenteritis,[3] and from spray-dried eggs.[4] According to Edwards (Enteric Bacteriology Laboratories, Communicable Disease Center), *S. kentucky* is found occasionally in infections of man and fowls but has never been isolated from mice or rats.

At the time of the isolation described above, there was no evidence of an epidemic due to *S. kentucky*, or any other Salmonella strain, in our laboratory mice colony. This lack of spread within a colony kept in crowded conditions was in contrast with the known high epizooticity of *S. typhimurium* and *S. enteritidis* and it was, therefore, decided to study the pathogenicity of *S. kentucky* for our laboratory mice.

It was found that intraperitoneal inoculation of an undiluted broth culture of *S. kentucky* killed adult albino mice in 18 hours or less, a fact which points to the presence of an active enterotoxin. Subcutaneous inoculation of the same broth culture led to death in three to four days; focal lesions were not observed in any organ. Attempts were then made to reproduce experimentally the natural disease by various routes of infection. Prolonged oral administration of *S. kentucky* by mixing a broth culture of the organism with the drinking water of the mice led to no apparent disease and the Salmonella could not be cultured from the liver and other viscera. Dropping a suspension of *S. kentucky* into the eyes of the mice produced conjunctivitis in a few cases, but there was no evidence of dissemination of the organism. Intranasal inoculation of 6 mice was followed by death in one to four days, with marked congestion of the lungs; 1 of the mice survived and, on being killed six weeks after the intranasal inoculation, *S. kentucky* could be cultured from the liver which, however, was free of macroscopic lesions.

Six other adult mice received an inoculation of 0.05 ml. of a broth culture of *S. kentucky* into one hindfoot pad. Marked swelling and hyperemia of the foot and the leg were observed and subsided about four weeks after the inoculation. These mice showed no other sign of disease, with the exception of 1 mouse which was prostrate and cachectic and died on the twenty-ninth day after inoculation; *S. kentucky* could be cultured from its liver, but no focal lesions were observed in this organ. One of the 5 other mice inoculated in the foot pad

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* The author is indebted to Dr. I. Olitzky, of the Philadelphia Department of Health, for his kind assistance in the serological identification of this organism.

was killed six weeks after inoculation; the liver showed no macroscopic pathological changes but contained *S. kentucky* in pure culture.

It is known that *S. typhimurium* and, to a lesser extent, *S. enteritidis* are capable of infecting laboratory mice when introduced by the oral or the conjunctival routes, a fact which explains the marked power of spread of these two species. As has been shown above, *S. kentucky* seems unable to establish infection (as distinguished from what seems to be purely toxic effects) unless it is introduced directly into the tissues. It is, therefore, to be expected that this organism will not be a cause of widespread typhoid in mice colonies.

The strain of *S. kentucky* isolated from the mouse was readily inhibited *in vitro* by chlortetracycline (Aureomycin), chloramphenicol (Chloromycetin), dihydrostreptomycin, and oxytetracycline (Terramycin).

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# Isolation and Identification of the Phenothiazine Urinary Conjugate in Dairy Cows

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URINARY ELIMINATION products of phenothiazine have been investigated in the sheep, horse, pig, dog, rabbit, rat, calf, and man.[3, 8]

DeEds *et al.*[7] reported elimination products of phenothiazine in the rat, rabbit, and in man after oral administration of the drug. Investigations in sheep were conducted by Lipson[9] and Swales *et al.*[10] Isolation and identification of a urinary conjugate from sheep, as the potassium salt of the ethereal sulfate of 3-hydroxyphenothiazine, was accomplished by Collier[5] and Collier, Allen, and Swales.[6] Benham[1] identified an elimination conjugate in rabbits as the glucuronic acid conjugate of thionol. A conjugate of phenothiazine and a polypeptide was identified in the urine of young calves by Clare *et al.*[4]

Few reports are available on the metabolism of phenothiazine in dairy cows. The investigation reported here concerns the isolation of a phenothiazine urinary elimination product in dairy cows and its identification as the potassium salt of the ethereal sulfate of 3-hydroxyphenothiazine.

## MATERIALS AND METHODS

Lactating Holstein-Friesian dairy cows were given 100-Gm. doses of phenothiazine, N.F. Their daily ration consisted of 5 lb. of ground grain and alfalfa hay offered ad libitum.

Urine was collected at intervals for the first 48 hours after administration of the drug. The urine was stored in a large carboy and held in a refrigerated room at 5 to 10 C. for three days to promote the formation of a bulky, light-colored precipitate. The bottle was tightly stoppered to reduce oxidation of the urine.

## RESULTS

*Isolation.*—The precipitated urine was filtered with the aid of Celite 503 upon a large Buchner funnel, at 5 to 10 C., to prevent excessive breakdown of the elimination product. The residue was extracted with ether to remove the elimination product from the filter aid. Trichloroacetic acid was added slowly to the ether extract to precipitate any proteinaceous materials which had been removed by filtration. The ether was evaporated from the filtrate and the residue was recrystallized four times from 95 per cent ethanol. The crystalline product was dried in a vacuum (under pressure, 15 lb./sq. in.) at 80 C. The final crystalization gave an excellent yield of sheaves of colorless needles.

*Identification.*—The melting point of the crystalline urinary elimination product was determined in a special melting point apparatus built in this laboratory. The temperature increment of the silicon oil bath used in the apparatus was controlled at the rate of a 2-degree (C.) rise per minute. The conjugate did not melt sharply but darkened between 216 to 220 C. On a Fisher-Johns melting point block, the conjugate darkened at 220 C. and decomposed without melting at 240 to 265 C. Samples of the purified bovine urinary product were subjected to elementary analysis.

Found.*—Carbon, 43.36; hydrogen, 2.47; nitrogen, 4.58; sulfur, 19.33; ash (as sulfate), 25.59.

Found.†—Carbon, 43.23, 43.37; hydrogen, 2.42, 2.49; nitrogen, 4.33, 4.32; sulfur, 19.11, 19.20; ash (as sulfate), 26.11, 26.20.

Calculated for $C_{12}H_8O_4NS_2K$.—Carbon, 43.22; hydrogen, 2.42; nitrogen, 4.20; sulfur, 19.23; ash (as sulfate), 26.13.

The infrared spectrum of the urinary conjugate (graph 1), in contrast to that of phenothiazine, showed several intense bands in the region 7.8 to 8.6 $\mu$ which are considered typical of sulfates. Otherwise, the spectrums of the two compounds are nearly identical, with the exception of minor differences attributable to substitution on the aromatic ring in the 3-position.

*Acid Hydrolysis of the Urinary Conjugate.*—Recrystallized bovine urinary conjugate (0.125 Gm.) was dissolved in 50 ml. of 5 per cent aqueous hydrochloric acid at room temperature and allowed to stand exposed to the air in a shallow evaporating

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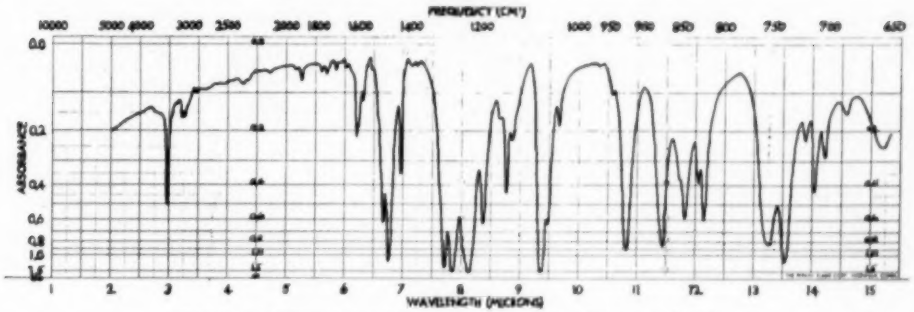



Graph 1—Infrared spectrum of the bovine urinary conjugate.

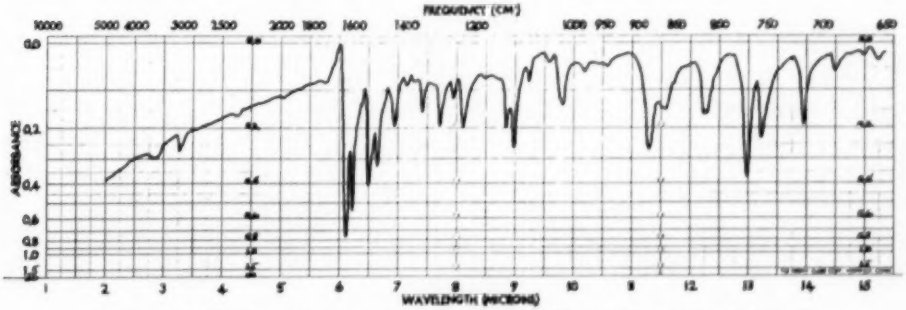
dish. The solution darkened within one hour and, after six hours, tiny black crystals began to separate from the deep purple solution. After three days, the product was collected by suction filtration. Its weight was 0.087 Gm., and its melting point 121 to 130 C.

The product was taken up in 25 ml. of reagent chloroform and poured on a column of 12.0 Gm. of Alcoa F-20 alumina. The chromatogram was developed with reagent grade chloroform. An intense purple band moved slowly down the column and into the effluent as a blood-red solution. The column was washed with 200 ml. of 50 per cent ethanol in chloroform. The combined effluents were evaporated to dryness on the steam bath under reduced pressure, yielding 0.07 Gm. of red-brown residue. The product was recrystallized from several milliliters of 50 per cent aqueous ethanol to obtain 0.02 Gm. of red-brown flakes which melted sharply at 159.0 to 159.5 C.

Ethereal sulfates as a class are readily cleaved, in the cold, by dilute mineral acids. If the bovine conjugate were the potassium salt of the ethereal sulfate of 3-hydroxyphenothiazine, the initial hydrolysis product would be 3-hydroxyphenothiazine which, under the conditions of this reaction, is known to readily undergo air oxidation to phenothiazone.

*Preparation of Phenothiazone.* A sample of crude phenothiazone (2.0 Gm.) was prepared according to Bernthsen,[2] and purified by passage through a column of 60 Gm. of Alcoa F-20 alumina, using reagent grade chloroform as solvent and developer. On the top of the column a broad black-brown band appeared from which the intense purple band of phenothiazone separated to move down the column and into the effluent. The effluent containing the purple band was concentrated and rechromatogrammed in the same manner, but this time only a trace of the brown band appeared. The blood-red effluent solution was evaporated to dryness under reduced pressure on the steam bath, yielding 0.3 Gm. of red-brown residue which melted over the range 145 to 155 C.

The product was recrystallized from boiling ethanol to yield 0.132 Gm. of red-brown flakes which melted at 155.5 to 156.5 C. The recrystallized product was




Graph 2—Infrared spectrum of the hydrolysis-oxidation product (phenothiazone).

dried for eight hours *in vacuo* over phosphorus pentoxide at 57 C.

Analysis, Calculated for $C_{12}H_7ON8$.—Carbon, 67.58; hydrogen, 3.31; nitrogen, 6.57; sulfur, 15.03. The calculated molecular weight of the product was 212.25.

Found.—Carbon, 66.70; hydrogen, 3.22; nitrogen, 6.62; sulfur, 15.52. The actual molecular weight found was Rast, 212.00.

The melting point of this product was not depressed when admixed with a sample of the product from the acid hydrol-

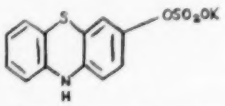



**Fig. 1—Bovine urinary conjugate—the potassium salt of the ethereal sulfate of 3-hydroxyphenothiazine.**

ysis of the urinary elimination product. A chloroform solution of 3 mg. each of this product and phenothiazone produced a single intense purple band ($R_f = 0.16$) when chromatogrammed on F-20 alumina with reagent grade chloroform as solvent.

The infrared and ultraviolet spectrums of the product from acid hydrolysis and air oxidation of the urinary conjugate are identical with those of phenothiazone (graph 2).

As a result of these studies, it is concluded that the bovine urinary conjugate is the potassium salt of the ethereal sulfate of 3-hydroxyphenothiazine (fig. 1).

## DISCUSSION

The bovine urinary conjugate of phenothiazine isolated in these studies is the same as the urinary conjugate obtained by Collier *et al.*[6] in their investigations in sheep.

The methods of detoxification of drugs in the animal body are chemical breakdown and elimination of the products in the urine and feces, conjugation with potassium sulfate, conjugation with glucuronic acid, and conjugation with polypeptides. The identification of the ethereal sulfate side chain of the 3-hydroxyphenothiazine made it possible to point out the site of conjugation as the liver. The presence of potassium instead of sodium in the conjugate structure may be due to conjugation in the intracellular phase of liver cells.

## SUMMARY

Purified bovine urinary conjugate of phenothiazine was isolated from the urine of lactating dairy cows fed 100.0 Gm. of phenothiazine, N.F.

The bovine urinary conjugate was identified as the potassium salt of 3-hydroxyphenothiazine ethereal sulfate.

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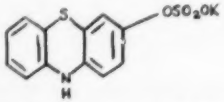


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